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Sabadilla Alkaloids VII*

Sabatine and Its Alkamine Sabine

By HYMAN MITCHNER and LLOYD M. PARKS†

A corrected molecular formula is proposed for the monoacetate alkaloid sabatine, $C_{29}H_{47-49}O_8N$, and its alkamine sabine, $C_{27}H_{45-47}O_7N$. It has been shown that sabine neither isomerizes nor possesses the masked α-ketol system present in other highly oxygenated alkamines isolated from Sabadilla or from other *Veratrum* species.

THE ALKALOID SABATINE was isolated in 1951 by Hennig, Higuchi, and Parks (1) as a constituent of *Schoenocaulen officinale*, more commonly called Sabadilla. Sabatine was reported to be a monoacetate ester of the alkamine sabine and was obtained with chloroform of crystallization present. Sabine was obtained in the crystalline form as a sulfate salt. Because of the nature of the crystalline materials it was not possible to report unequivocal analytical data and this complicated the determination of empirical formulas and further characterization of the two substances. Molecular formulas approximating $C_{31}H_{53}O_9N$ for sabatine and $C_{29}H_{51}O_7$ for sabine were postulated.

In the present study of sabatine and sabine crystallized from solvents other than those used previously, new data have indicated that sabine is probably a C_{27} alkamine ($C_{27}H_{45-47}O_7N$). The C_{27} carbon skeleton appears to be common to the alkamines isolated from *Veratrum* species (2). All of the other alkaloids previously isolated from Sabadilla, veratridine, cevadine, cevacine, and vanilloylveracevine were esters of the alkamine veracevine, $C_{27}H_{43}O_8N$. Veracevine in common with the other highly hydroxylated alkamines: germine, protoverine, and zygadenine possesses a masked α-ketol system capable of undergoing isomerization (2, 3). However, hydrolysis experiments and countercurrent distributions indicate that sabine is unique in the highly oxygenated alkamines in that it appears to be a stable alkamine, incapable of undergoing isomerization and therefore probably lacking a masked α-ketol system.

EXPERIMENTAL

Sabatine from Acetone-Water.—A 1-Gm. sample of sabatine, crystallized from chloroform, was dissolved in a small volume of acetone. Water was added until the solution became cloudy. Upon warming and adding a few drops of acetone the solution cleared, and after being allowed to stand overnight 700 mg. of crystalline sabatine was obtained which did not give a chlorine test upon fusion with sodium. The sabatine was dried at 100° under vacuum for sixteen hours, m. p. 256–258°.

	$C_{29}H_{47}O_8N \cdot {}^1/_2H_2O$ Calcd.	$C_{29}H_{49}O_8N \cdot {}^1/_2H_2O$ Calcd.	Found
C	63.71	63.48	63.45, 63.81
H	8.85	9.19	9.19, 9.06

Sabatine from Ethyl Acetate.—Five hundred milligrams of sabatine, crystallized from acetone-water, was dissolved in 50 ml. of ethyl acetate. The solution was evaporated to 10 ml. and placed in a refrigerator. After two days, 410 mg. of crystalline sabatine was obtained, m. p. 256–258°.

	$C_{29}H_{47}O_8N$ Calcd.	$C_{29}H_{49}O_8N$ Calcd.	Found
C	64.78	64.52	64.42, 64.79
H	8.82	9.15	8.75, 8.84

* Received January 17, 1959, from the Department of Pharmaceutical Chemistry, University of Wisconsin, Madison.

Supported in part by the Research Committee of the University of Wisconsin from funds supplied by the Wisconsin Alumni Research Foundation.

† Present Address: College of Pharmacy, Ohio State University, Columbus.

Volatile Acid Determination on Sabatine.—This was carried out on 30-mg. samples of the pure alkaloid by the procedure of Niedrl and Niedrl (4), and in two determinations it showed 0.96 and 0.98 moles of a volatile acid. As a check upon the complete hydrolysis of the alkaloid, the procedure of Nash and Brooker (5) was also used and showed only 1 mole of a volatile acid.

Investigation of the Acids of Sabatine.—A sample of sabatine (27.8 mg.) was hydrolyzed with 1 ml. of *p*-toluenesulfonic acid as in the volatile acid determination. The hydrolysate was washed into a 50-ml. beaker with 1 ml. of water, thoroughly triturated with 2 Gm. of silicic acid, and slurried with chloroform. The slurry was added to the top of a previously prepared partition column (internal phase 20 ml. of water plus 20 Gm. of silicic acid; chloroform as the external phase). The column was developed using a series of solvents increasing in polarity as suggested by Marvel and Rands (6). Fractions of 8 ml. were collected and, after the addition of 1 ml. of water and a drop of *m*-cresol purple indicator, titrated with 0.00976 *N* sodium hydroxide. Fraction 21–32 showed an acid content corresponding to 0.93 moles of acid. No other acidic material was found in the 72 fractions collected.

The acidic fractions, 21–32, were combined and the chloroform and aqueous phases were separated. The chloroform was washed with 5 ml. of 0.01 *N* sodium hydroxide and the washings added to the combined aqueous phases. *p*-Toluenesulfonic acid was added to the aqueous solution until it became acid to litmus. The solution was then distilled and the first 30 ml. of the distillate was collected. A micro Duclaux determination was performed on the distillate. Four-milliliter fractions were collected and titrated with 0.00976 *N* sodium hydroxide. The Duclaux values obtained were 6.48, 7.07, 7.37, corresponding to acetic acid.

Hydrolysis of Sabatine and the Isolation of Sabine

Hydrolysis with Sodium Ethoxide.—To a solution of 200 mg. of sodium in 30 ml. of absolute ethanol 200 mg. of sabatine was added. The solution was heated under reflux for four hours. After cooling, the hydrolysis mixture was adjusted to a pH of 6.5 with tenth normal hydrochloric acid and the ethanol distilled *in vacuo*. The concentrate was made strongly alkaline with 5 *N* potassium hydroxide and extracted with three 25-ml. portions of chloroform. The chloroform was dried over anhydrous sodium and evaporated to dryness *in vacuo* on a steam bath. The residue was taken up in 30 ml. of hot ether, filtered, and evaporated to about 5 ml. On standing, 135 mg. of large cubic crystals formed which were contaminated with a brown color. The crystalline material was difficultly soluble in ether, and for recrystallization it was necessary to dissolve it in a small volume of chloroform and evaporate under reduced pressure to dryness on a steam bath before dissolving the material in hot ether. The crystals frothed at 105–110° and melted at 173–176°. The infrared spectrum indicated that an ester band was not present (Fig. 1). The molecular weight studies (Table II) indicated that solvent of crystallization must have been incorporated.

	$C_{27}H_{45}O_7N \cdot C_4H_{10}O$ Calcd.	$C_{27}H_{47}O_7N \cdot C_4H_{10}O$ Calcd.	Found
C	65.49	65.11	65.09, 65.11
H	9.77	10.04	9.62, 9.81

Upon drying at 117° under reduced pressure, the sabine from ether lost its shiny appearance. The

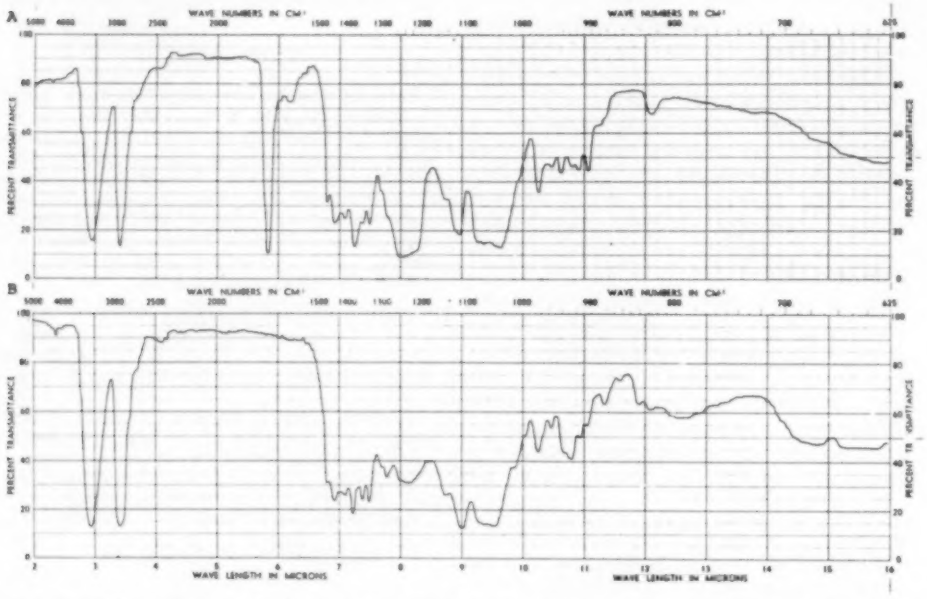

Fig. 1.—Infrared spectra of: (A) sabatine in chloroform solution and (B) sabine in chloroform solution.

infrared spectrum of this material was almost identical with the sabine from ether that was dried at 100°. However the sabine dried at this higher temperature no longer foamed at 105–110°, but still melted at 173–176°. The molecular weight (Table II) was considerably lower after drying at 117°.

	$C_{27}H_{43}O_7N$ Calcd.	$C_{27}H_{45}O_7N$ Calcd.	Found
C	65.42	65.16	65.09, 65.24
H	9.16	9.52	8.85, 9.10

Mild Alkaline Hydrolysis.—An 870-mg. sample of sabatine was dissolved in 25 ml. of methyl alcoho, and 5 ml. of 1 *N* sodium hydroxide. The solution was refrigerated at 0° for twenty-four hours and then distilled under reduced pressure to about 5 ml. The concentrate was extracted with ten 5-ml. portions of chloroform. After combining the chloroform extracts, the chloroform was washed with 5 ml. of water, dried over anhydrous sodium sulfate, and evaporated under reduced pressure to dryness on a steam bath. The residue was taken up in 40 ml. of hot ether and filtered. On cooling, 577 mg. of crystalline material was obtained which was identical in all respects to the sabine isolated from the sodium ethoxide hydrolysis.

Methanolysis of Sabatine.—A 300-mg. sample of sabatine was treated in a similar manner to the procedure used for the methanolysis of cevacine (7). The sabatine was dissolved in 10 ml. of methanol and 5 ml. of water, and allowed to stand at room temperature for twenty hours. The methyl alcohol and water were distilled *in vacuo*. The residue was dissolved in 40 ml. of chloroform which was boiled to dryness to remove any traces of water. All attempts to crystallize the white residue were unsuccessful. The infrared spectrum of the material showed the presence of an ester band but other peaks were not distinct.

Countercurrent Investigation of Sabatine Hydrolysis Mixtures

From Sodium Ethoxide Hydrolysis.—The reaction mixture analyzed was the ether mother liquor remaining after removal of the crystalline sabine in the sodium ethoxide hydrolysis as described above. The ethereal solution was evaporated to dryness and the residue was dissolved in 10 ml. of chloroform. This chloroform solution was distributed for 65 transfers in a Craig[1] countercurrent apparatus. The lower phase was chloroform and the upper phase was 0.5 *M*, pH 8.1 phosphate buffer, with 10 ml. of each phase per tube. The alkaloid was analyzed by a nonaqueous titration procedure (8). Figure 2 shows the separation obtained. The tubes containing the peaks were collected and combined in two fractions. The aqueous phases were made very strongly alkaline with 5 *N* potassium hydroxide and thoroughly shaken. The chloroform phases were then withdrawn, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. The amount of alkaloidal material from each peak was insufficient for crystallization but their infrared spectra were identical to sabatine and sabine for the first and second peaks, respectively.

A second sodium ethoxide hydrolysis was carried out on 200 mg. of sabatine. However, after extracting the total alkaloidal components in chloroform, the chloroform solution was evaporated to 10 ml. and distributed for 57 transfers in the Craig countercurrent apparatus. Chloroform was again used as the lower phase and 0.5 *M*, pH 8.15 phosphate buffer was used as the upper phase. Tubes 39–57 were combined and 25 ml. of 5 *N* potassium hydroxide was added. After shaking and removing the chloroform, the aqueous layer was washed with a 200-ml. portion of chloroform. The combined chloroform solutions were dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The residue was dissolved in 40 ml. of hot ether and evaporated to about 10 ml. On standing, large cubic crystals formed which were identical to sabine in all respects. Sabatine was present in the first peak but only in a very small amount.

From Methanolysis.—A 200-mg. sample of the methanolysis reaction mixture isolated as described above was dissolved in 10 ml. of chloroform and distributed in a Craig countercurrent apparatus for 100 transfers. Chloroform was used as the lower phase and 0.5 *M*, pH 8.1 phosphate buffer as the upper phase. The distribution obtained is shown in Fig. 3. Tubes 0–30 and 70–100 were combined in two separate fractions. To each fraction 50 ml. of 5 *N* potassium hydroxide was added and the phases shaken. The chloroform phases were removed and the aqueous phases were each shaken with two 100-ml. portions of chloroform. The chloroform washings were combined with their respective original chloroform extracts, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. The alkaloidal material from tubes 70–100 was taken up in hot ether, filtered, and evaporated to about 5 ml. On standing, cubic crystals were obtained for which analytical data agreed in all respects with sabine. The alkaloidal material from tubes 0–30 was taken up in a very small volume of chloroform and seeded with sabatine previously isolated from chloroform solution. On standing, needle crystals were obtained which were identical in all respects to sabatine.

Reaction of Sabine, Sabatine, Cevadine, Cevacine, Veracevine and Its Isomers With Triphenyltetrazolium Chloride.—Five milligrams each of sabatine, sabine, veracevine, cevagenine, cevine, cevacine, and cevadine was placed in separate test tubes, and to each was added 1 ml. of a 1% aqueous triphenyltetrazolium chloride solution and 1 ml. of 0.1 *N* sodium hydroxide. A control was prepared with the two solvents alone. The solutions were allowed to stand at room temperature and were observed at various time intervals to note any color changes. After seventy-two hours the solutions of cevine, sabatine, sabine, and the control were sealed in ampuls and heated at 100°, again noting any color changes. The results are expressed in Table I.

Periodic Acid Titration of Sabatine and Sabine.[2]—Samples of sabatine (19.0 mg.) and sabine (20.8 mg.) were dissolved in a solution of 10 ml. of 0.05 *M* periodic acid and 5 ml. of 5% acetic acid, and then

[1] The Craig apparatus used was a 200 plate, robot driven instrument, manufactured by H. O. Post, Scientific Instrument Co., Maspeth, N. Y.

[2] This titration was kindly performed by Dr. M. Neeman of the Chemistry Department, University of Wisconsin.

TABLE I.—REACTION OF TRIPHENYLTETRAZOLIUM CHLORIDE WITH CEVADINE, CEVACINE, VERACEVINE CEVAGENINE, CEVINE, SABATINE, AND SABINE

	Reaction at 25°							
	5 min.	30 min.	2.5 hr.	6 hr.	12 hr.	24 hr.	48 hr.	72 hr.
Cevadine	...	...	+	+	+	++	+++	+++
Cevacine	...	+	++	++	+++	+++	+++	+++
Veracevine	+	++	+++	+++	+++	+++	+++	+++
Cevagenine	+++	+++	+++	+++	+++	+++	+++	+++
Cevine	...	...	...	+	+	+	+	+
Sabatine	...	...	...	...	...	...	...	...
Sabine	...	...	...	...	...	...	...	...
Control	...	...	...	...	...	...	...	...

Reaction at 100° (A Continuation of the Above Reaction But in Sealed Ampuls)

	30 min.	6 hr.	12 hr.	24 hr.	48 hr.	72 hr.
Cevine	+++	+++	+++	+++	+++	+++
Sabatine	...	...	...	Yellow	Yellow	Yellow
Sabine	...	...	...	Yellow	Yellow	Yellow
Control	...	...	...	Yellow	Yellow	Yellow

+ Very faint pink color. ++ Definite pink color. +++ Red color.

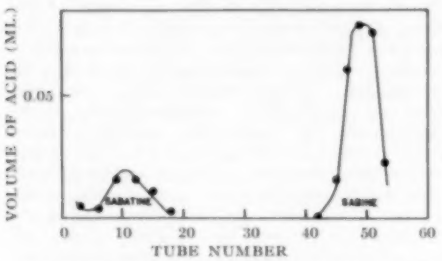

Fig. 2.—Countercurrent distribution of the alkaloidal constituents in the mother liquor from the sodium ethoxide hydrolysis of sabatine after the removal of crystalline sabine—chloroform *vs.* pH 8.1 phosphate buffer.

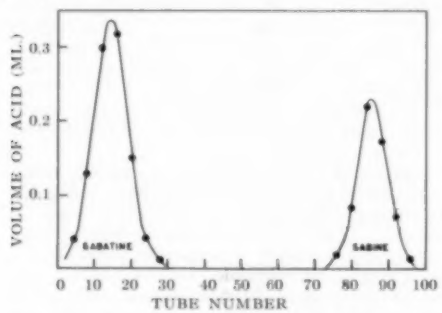

Fig. 3.—Countercurrent distribution of the products from the methanolysis of sabatine—chloroform *vs.* pH 8.1 phosphate buffer.

diluted to 25 ml. At time intervals of one hour and fifteen minutes, two hours and thirty minutes, and twenty-six hours, 5-ml. aliquots of the reaction mixture were withdrawn, treated with 5 ml. of 0.06 *N* sodium arsenite, 5 ml. of a saturated aqueous solution of sodium bicarbonate, and titrated with 0.02 *N* iodine solution. The periodic acid consumed by the sabine corresponded to 1.9 moles, the acid consumed by the sabatine to 0.9 moles at all time intervals.

Equivalent Weight Titrations of Alkamines and Alkaloids.—Samples of alkaloidal substances of from 4 to 10 mg. were dissolved in 4 ml. of reagent grade chloroform. To this solution, three drops of thymol blue indicator (0.1% solution in butyl alcohol) were added and the solution titrated with 0.01122 *N* perchloric acid in dioxane to the permanent appearance of a pink color, the color changing from yellow to pink. The perchloric acid was standardized by a similar titration in chloroform against reagent diphenylguanadine. The results are summarized in Table II.

DISCUSSION

The chloroform of crystallization in sabatine crystallized from chloroform can be removed by recrystallization from acetone-water. However, elemental analyses and molecular weight determinations indicated that, even after drying at 100° the sabatine from acetone-water contained water of crystallization. Upon recrystallization from ethyl acetate the water was removed and solvent-free sabatine which melted at 256–258° was obtained, and for which the formula $C_{29}H_{47-49}O_8N$ was in agreement with elemental analyses and molecular weight determinations. A volatile acid determination on sabatine and chromatography of the hydrolytic products indicated that sabatine was indeed a monoacetate ester as it had already been suggested (1).

Hydrolysis of sabatine with hot sodium ethoxide or with cold methanol and sodium hydroxide yielded an alkamine fraction which could be crystallized from ethyl ether to give a material which frothed at 105–110° and melted at 173–176°. The infrared spectrum of this material showed no trace of an ester band (Fig. 1). In almost all respects it appeared to be identical to the alkamine sabine that Hennig, Higuchi, and Parks had obtained previously in an amorphous form from ether (1). The molecular weight of the sabine crystallized from ether was greater than the molecular weight of the parent ester sabatine (Table II). It appeared that the alkamine had taken on solvent of crystallization. If the sabine was dried under reduced pressure at a temperature of 117°, the alkamine lost its shiny appearance and gave a material which did not froth at 105–110° but did melt at 173–176°. The infrared spectrum for the sabine before drying at 117° was almost identical to the infrared spectrum

TABLE II.—EQUIVALENT WEIGHT TITRATIONS OF ALKAMINES AND ALKALOIDS

Material	Formula	Wt. of Sample, mg.	Equivalent Wt. Calcd.	Found
Veracevine	$C_{27}H_{43}O_8N$	6.05, 4.94	510	515, 516
Cevine	$C_{27}H_{43}O_8N$	7.92, 9.07	510	509, 517
Germine	$C_{27}H_{43}O_8N$	5.11, 6.96	510	508, 505
Sabatine (from ethyl acetate)	$C_{29}H_{47-49}O_8N.{}^1/_2H_2O$	6.34, 7.86	538–540	533, 536
Sabine (from ether)	$C_{27}H_{45-47}O_7N \cdot C_4H_{10}O$	8.00, 5.40	570–572	558, 562
Sabine (after drying at 117°)	$C_{27}H_{45-47}O_7N$	6.41, 6.10	496–498	496, 500

after drying. The molecular weight of the sabine after drying corresponded to that which would have been expected for sabine based on the loss of an acetate group from the proposed sabatine formula (Table II). The elemental analyses and molecular weight determinations were in good agreement for the formula $C_{27}H_{45-47}O_7N$.

It has been shown that many of the alkamines such as germine, veracevine, protoverine, and zygadenine (2, 3) undergo an alkali induced isomerization. There existed the possibility that sabine, with a very similar molecular formula to the above alkamines, might undergo similar isomerization. Following the crystallization of sabine from the sodium ethoxide hydrolysis of sabatine, the alkaloidal content of the mother liquor was recovered and investigated with a 65-transfer countercurrent distribution as shown in Fig. 2. Only two peaks were obtained and these corresponded to unhydrolyzed sabatine and to sabine. Methanolysis of sabatine gave a mixture that could be resolved by countercurrent distribution only into two peaks from which sabatine and sabine could be isolated (Fig. 3). No indication of any isomeric alkamines was obtained under either the strong or the mild hydrolytic conditions.

Auterhoff and Kraft (9) have shown that the masked hemiketol systems which occur in veracevine and germine can be reduced with triphenyltetrazolium chloride to produce a red reaction product. Under conditions such that cevadine, the angelic acid ester of veracevine, and the isomeric alkamines, veracevine, cevagenine, and cevine, all reacted with the triphenyltetrazolium chloride, sabatine and sabine failed to reduce the reagent. This failure and the presence of only a single hydrolysis product under both strong and mild hydrolytic conditions suggested not only that sabine is the parent and only alkamine of the ester sabatine but also that the masked hemiketol system found with veracevine and germine (3) is absent in sabine.

Periodic acid titration of sabine showed a take-up corresponding to 2 moles of the acid, whereas sabatine consumed only 1 mole. The acetate group in sabatine could be assumed to be blocking one of the adjacent hydroxyl pairs.

The tendency of sabatine and sabine to pick up solvent of crystallization placed a great amount of stress on the analytical data that could be obtained and particularly on molecular weight determinations. With the small samples of alkaloidal material used in molecular weight studies, nonaqueous potentiometric titrations did not give consistent results. However, a nonaqueous titration procedure similar to the one used in the analysis of countercurrent distributions (8) was found to give results with less than a 2% error. This was checked with pure alkamine samples of known constitution. All molecular weight determinations have been summarized in Table II.

SUMMARY AND CONCLUSIONS

1. It has been confirmed that sabatine, an ester alkaloid isolated from Sabadilla, is a monoacetate ester. New analytical data on the crystalline alkaloid suggested corrected molecular formulas of $C_{29}H_{47-49}O_8N$ for sabatine and $C_{27}H_{45-47}O_7N$ for the alkamine sabine.

2. Under both mild and strong hydrolytic conditions, sabatine yielded only the one alkamine, sabine. Both sabatine and sabine failed to reduce triphenyltetrazolium chloride reagent. The failure of the alkamine to isomerize or to reduce the TTC reagent indicated the absence of a masked α-ketol structure that has been found in the other highly oxygenated alkamines isolated from Sabadilla and other *Veratrum* species.

3. There exists two adjacent hydroxyl pairs in sabine that are susceptible to periodate oxidation and only one pair in sabatine, indicating that the acetate ester group is blocking one of these hydroxyl pairs.

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A Radiometric Method for Determination of Absorption of Ammoniated Mercury from Ointments*

By DONALD L. SORBY and ELMER M. PLEIN

Ammoniated mercury-Hg^{203} was prepared from mercuric chloride-Hg^{203}. The labeled ammoniated mercury was incorporated into ointment bases and used to determine absorption of ammoniated mercury through intact and abraded skin of rats. Twenty-four hours after application of the ointment, both kidneys were removed and assayed for mercury content by beta and gamma radiometric procedures. The two radiometric methods were found to give comparable results.

THE ANALYTICAL DETERMINATION of a medicinal substance in tissues of laboratory animals following absorption from an ointment base is often difficult due to the small amounts of medicinal substance encountered and to the presence of interfering biological material.

Radioisotopes have been used by several investigators to study drug absorption through the skin. The following are representative works of this type of study. Cyr, *et al.* (1), Skauen, *et al.* (2), and Plein and Plein (3) used sodium radioiodide-I^{131} to determine absorption of sodium iodide from various ointment bases. Neesby, *et al.* (4), used sulfur-S^{35} to determine absorption from polythionate solutions applied to the skin of laboratory animals. Wernsdörfer (5) determined the amount of absorption of mercuric chloride-Hg^{203} through intact skin of guinea pigs.

In many methods, gamma-emitting isotopes have been used in determining the amount of medicinal substance in tissue. Often it is desired to study an isotope which emits beta radiation only. Self-absorption of beta particles by the tissue thus becomes a problem which is not experienced in counting gamma radiation. Digestion of tissue samples is then necessary, but is often tedious and time-consuming.

In this experiment it was desired to introduce a simple and rapid method for determining the amount of beta-emitting substance in tissue. Mercury-Hg^{203} was selected for two reasons. Because it emits both beta and gamma radiation, results obtained by beta methods could be conveniently verified by gamma counting of samples. Work of other investigators (6–10) has shown that the usual colorimetric procedures for determining mercury[1] in tissue are difficult and not of the desired selectivity (3) in the concentration ranges encountered. It was expected that the radiometric method would be more favorable than usual methods for studying absorption of mercurials and would give more nearly correct results.

EXPERIMENTAL

The method of Leblond, *et al.* (11), for the assay of beta-emitting isotopes in tissues was adapted for determination of the percutaneous absorption of mercury-Hg^{203} from ointments.

Preparation of Labeled Ammoniated Mercury.—Mercury-Hg^{203} was obtained as a solution of mercuric chloride containing in each ml. 17.5 mg. of mercuric chloride and 1.5 mc. of radioactivity. For preparation of ammoniated mercury-Hg^{203}, 1.5875 Gm. mercuric chloride (reagent grade) was dissolved in 25 ml. of warm water and cooled. Mercuric chloride-Hg^{203} solution (6.8 ml.) was added and the resulting solution poured, with stirring, into 5.2 ml. of cold 10% ammonium hydroxide. The precipitate was filtered from solution, washed with 40 ml. of 0.5% ammonium hydroxide solution, and dried in a desiccator. The isotope yield was nearly 100%.

Preparation of Standard Solution of Ammoniated Mercury-Hg^{203}.—A solution was prepared by dissolving 49.5 mg. of ammoniated mercury-Hg^{203} in 1.5 ml. of concentrated hydrochloric acid and adding distilled water to make a volume of 10 ml. One ml. of the solution made to a volume of 100 ml. with distilled water produced a dilution which contained 39.4 μg. mercury per ml. This diluted solution was used as the standard for all mercury determinations.

Preparation of Ointments and Application to Laboratory Animals.—Three ointments were prepared for application to laboratory animals. Each ointment contained ammoniated mercury-Hg^{203} (5%) and liquid petrolatum (3%). The base for ointment A was white ointment, U. S. P. XV; for ointment B, hydrophilic petrolatum, U. S. P. XV; and for ointment C, the base contained stearyl alcohol (3%), white wax (8%), wool fat (15%), and white petrolatum (74%). Ointments were also prepared for use on control animals. For these ointments an equivalent amount of ointment base was substituted for the ammoniated mercury-Hg^{203}.

These ointments were applied to rats following the procedure of Plein and Plein (3). Twenty-four hours after application of the ointment the kidneys were removed for determination of mercury content.

According to Laug, *et al.* (7), mercury accumulates predominantly in the kidneys following percutaneous absorption. It was recommended that

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[1] The term "mercury" is used to describe all chemical combinations of mercury in tissues. All calculations were based on the quantity of atomic mercury rather than its salts.

the quantity of mercury appearing in the kidneys twenty-four hours after application of the ointments be used as an index of the relative amounts of absorption from different ointments.

Preliminary Preparation of Samples for Counting.—The kidneys from each rat were ground together to a fine state of suspension using an A. H. Thomas teflon pestle tissue grinder. The suspensions were made to a 10-ml. volume with distilled water and refrigerated until used for assay. The suspensions produced in this manner were fine enough to flow from a serological pipet.

Determination of Mercury Content of Samples from Gamma Activity.—The kidney samples were prepared for gamma counting by pipetting 1 ml. of tissue suspension into a 1-dram screw-cap vial. The activity of the samples was counted with a well type scintillation counter.[2] The mercury content of the sample was determined by comparing its activity with the activity of a known quantity of standard ammoniated mercury-Hg^{203} solution.

Determination of Mercury Content of Samples from Beta Activity.—One ml. of the kidney tissue suspension was pipetted into each of two fired stainless steel cup type, Tracer Lab E-20 1 inch by $^5/_{16}$ inch, planchets. One-tenth milliliter of standard ammoniated mercury-Hg^{203} solution was added to one planchet and 0.2 ml. of 1% sodium sulfide ($Na_2S \cdot 9H_2O$) solution was added to both planchets. The preparations were mixed by blowing air through a pipet onto the surface and were set aside to dry. To determine the activity of a known amount of mercury-Hg^{203}, 0.1 ml. of standard ammoniated mercury-Hg^{203} solution was pipetted into each of three fired planchets and 0.2 ml. of the 1% sodium sulfide solution was added to each planchet. When all samples appeared dry, they were dried for an additional twelve to twenty-four hours in a desiccator. The activity of the samples was then determined,[3] corrected for self absorbance and background, and compared with the activity of the standard solution. Following is a sample calculation of the mercury content of a kidney tissue suspension.

Suspension + added standard (standard = 1,500 c. p. m.)	= 3,000 c. p. m.[4]
Suspension	= 2,000 c. p. m.
Activity of added standard in the sample	= 1,000 c. p. m.

Activity of suspension corrected for self absorbance: $(1{,}000/1{,}500) = (2{,}000/X)$; $X = 3{,}000$ c. p. m.

Amount of mercury in the total sample: $3.94 \times (3{,}000/1{,}500) \times 10 = 78.8$ μg.

Absorption Studies on Rats with Ammoniated Mercury-Hg^{203}.—White, female Sprague-Dawley rats weighing from 180.5 to 220 Gm. were treated as described above. Ointments A, B, and C were used for intact skin studies and an abraded skin study was also carried out using ointment A. Each medicated ointment was applied to a group of six rats and in each series a control ointment was applied to a seventh rat.

The mercury content of the kidneys from each rat was determined by gamma counting and by beta counting. The results of these studies are summarized in Table I.

TABLE I.—RESULTS OF ABSORPTION STUDIES ON RATS WITH AMMONIATED MERCURY-Hg^{203}

Ointment	Mean Absorption in μg. Beta Determination	Mean Absorption in μg. Gamma Determination
A (Intact skin)	8.6 ± 2.0[a]	8.7 ± 2.1
A (Abraded skin)	39.0 ± 12.0	41.3 ± 12.4
B	9.2 ± 2.9	9.4 ± 3.1
C	10.5 ± 1.9	10.8 ± 2.3

[a] Standard deviation of the mean of six kidney samples was calculated by the formula $s = \sqrt{(1/n - 1)\Sigma d^2}$.

DISCUSSION

Beta counting of tissue samples was complicated by the volatility of mercurial compounds. Addition of 0.2 ml. of 1% sodium sulfide ($Na_2S \cdot 9H_2O$) solution to the planchets was found to be the most satisfactory method for reducing the volatility.

An attempt was made to determine the amount of penetration of mercury into the skin from the area of application of the ointment. The procedure used for the beta determination of mercury in the kidney samples was found to be unsatisfactory for use with the skin samples. It was thought that nonuniform sampling caused failure in agreement of the gamma and beta determinations of the skin samples. This nonagreement was probably due to inability to reduce the skin to a fine suspension using the teflon pestle tissue grinder. Heating the skin samples with a few drops of 90% hydrogen peroxide produced more uniform suspensions. After destroying the excess peroxide with 3% potassium permanganate, the radiometric assay was carried out and gave more favorable results. It is probably necessary to adjust the method of producing a uniform sample to the particular tissue being studied. Once a uniform sample is obtained, the general method for determination of the amount of isotope should be applicable.

A rather large variation was occasionally encountered between the amounts of absorption for different rats receiving the same type of ointment. These variations were also reported by other authors (3, 7–9).

Statistical analysis of the results was carried out using the t-test for determination of significant differences between sample means. The level of significance was chosen as $P = 0.10$. The results obtained by beta counting were compared with results of gamma counting of the same kidney samples. No significant differences were found between the two methods for the intact skin studies on ointment A, ointment B, and ointment C ($P > 0.50$). The

[2] The gamma activity of the samples was counted at 1,150 v. in a Tracer Lab P-20 scintillation detector with a 1-in. NaI(Tl) crystal connected to a Tracer Lab Autoscaler.

The authors wish to thank the Anatomy Department of the University of Washington for use of scintillation counting equipment.

In this research all samples were counted for a sufficient number of counts to have probable error in counting of 2% or less. Whenever necessary, corrections were made for decay loss. Duplicate determinations were carried out on all samples. The determination was repeated if there was nonagreement between duplicates of the same sample.

[3] The beta activity of the samples was determined at 1,400 v. with a Tracer Lab TGC-2 end window (1.6 mg./cm.^{2}) Geiger-Müller counter connected to a Tracer Lab SC-51 scaler equipped with a Tracer Lab SC-42 preset timer.

[4] Counts per minute referred to as c.p.m.

results of beta counting of mercury absorbed from ointment A through abraded skin of rats differed significantly from results obtained by gamma counting ($0.05 > P > 0.01$).

CONCLUSIONS

The radiometric method for determining absorption of ammoniated mercury through intact and abraded skin is simple and is accurate for determining small amounts of mercury encountered under the conditions of this type experiment. The procedure is suitable for testing dermatologic vehicles for their effect on absorption of drugs. The general method for beta determination is probably applicable to any mercurial used in ointment form and, with certain modifications, could be adapted to isotopes of other medicinals commonly studied for their release from ointment bases.

Since the gamma-counting method is uncomplicated by volatility and self-absorbance and since it is relatively easy to carry out, it is preferred to the beta-determination method. The beta technique requires less expensive equipment and would be advantageous in cases when isotopes emit only beta radiation.

The radiometric procedure is more simple than colorimetric methods and requires less sample treatment in carrying out the determination of mercury. The results confirm that the method of Leblond, *et al.* (11), is advantageous in studies of percutaneous absorption of medicinals and the theory that a radioactive tracer technique for determining absorption of ammoniated mercury is practical for use.

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Interaction of Preservatives With Macromolecules II*

Correlation of Binding Data With Required Preservative Concentrations of *p*-Hydroxybenzoates in the Presence of Tween 80

By F. D. PISANO and H. B. KOSTENBAUDER

Data are presented to show that the preservative activity of *p*-hydroxybenzoic acid esters in the presence of Tween 80 is primarily a function of the concentration of unbound preservative. Prediction of required preservative concentrations from a knowledge of the degree of interaction of the preservative with nonionic surfactants is illustrated.

A RATHER HIGH degree of association between *p*-hydroxybenzoic acid esters and Tween 80 has previously been demonstrated (1), and several investigators (2, 3) have suggested that such an interaction might account for the observed interference of nonionic surfactants with the preservative acitivity of the parabens and other phenolic preservatives. Although there is ample evidence that such inhibition does occur (2–10), the majority of these studies have not been designed in a manner which would permit a direct correlation of preservative activity and the degree of binding of the preservative by the surfactant.

Aoki, Kamata, Yoshioka, and Matsuzaki (9) presented data which indicated a relationship between solubility of the parabens in Tween 20[1] solutions and antifungal activity in these solutions. Aoki, Kamata, Matsuzaki, and Nakatani (11) conducted further studies on this system, using very dilute solutions of Tween 20 (<0.1%). In these dilute solutions there is little binding of the preservative, and the decrease in surface tension produced by the Tween may become a relatively important factor in determination of the required preservative concentration.

In the present investigation minimum inhibitory concentrations of methylparaben were obtained for *Aerobacter aerogenes* and *Aspergillus niger* in control media and in media containing several concentrations of Tween 80.[2] The

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[1] Tween 20 is polyoxyethylene sorbitan monolaurate, Atlas Powder Co., Wilmington, Del.
[2] Tween 80 is polyoxyethylene sorbitan monooleate, Atlas Powder Co., Wilmington, Del.

increased quantity of paraben required in the presence of Tween 80 was compared with the concentration which would be predicted from a knowledge of the degree of binding of the methylparaben by the Tween, and the assumption that the preservative activity is a function of the concentration of the unbound or free paraben.

A simple, chemically defined culture medium was chosen for these studies to avoid, so far as possible, any competing interactions of the preservative with components in the medium. Special precautions were taken with *Aspergillus niger* because, as noted in a report by Barr and Tice (12), this organism is capable of hydrolyzing the ester linkage in Tween, with liberation of the free fatty acid. In order to investigate any possible direct influence such as this, the minimum inhibitory concentration for *Aspergillus niger* was observed when the organism was in contact with the Tween and this hydrolysis was observed to proceed, and also when the organism was separated from the Tween by a nylon membrane and the hydrolysis was thus prevented.

EXPERIMENTAL

Reagents.—Recrystallized methyl *p*-hydroxybenzoate,[3] m. p. 129–131°; Tween 80, a commercial sample; all other chemicals were reagent grade.

Microorganisms.—The organisms selected for these studies were *Aerobacter aerogenes*[4] and *Aspergillus niger*.[5] It was necessary that the organisms chosen should be capable of growth in synthetic media and have an optimum growth temperature of about 30°, since binding data were readily available at this temperature.

Aerobacter aerogenes, in addition to satisfying the previous conditions, has the further advantage that growth can readily be followed either by titration of acid produced or by turbidity measurements.

Selection of the Culture Medium.—Evidence indicating binding of phenols by amides (13) and nylon (1) suggests the possibility that when studies are carried out using common media containing substances such as peptone or beef extract, some of the drug being studied may be bound to the protein or amino acids in the media. To avoid such interactions the following medium was selected:

Dextrose, anhydrous	5.00%
NaCl	0.50%
$MgSO_4.7H_2O$	0.02%
$K_2HPO_4.3H_2O$	0.10%
$NH_4H_2PO_4$	0.10%
Distilled water to make	100%

pH of medium 6.5.

The possibility of an interaction between methylparaben and dextrose was investigated by determining the solubility of methylparaben in dextrose solutions of varying concentration. It was found that there was no increase in methylparaben solubility in solutions containing up to 10% dextrose, and it was thus concluded that there was not a significant interaction between dextrose and the preservative.

Sterilization.—It was found that sterilization could be effected without caramelization of the dextrose if the salt solution was autoclaved separately and then added aseptically to the solution of dextrose, Tween, and paraben previously sterilized by autoclaving at 15 pounds for twenty minutes. It was also found that caramelization did not occur if the solutions were combined and then autoclaved at 15 pounds for fifteen minutes, followed by rapid cooling.

The possibility of hydrolysis of methylparaben upon autoclaving was investigated by determining the ultraviolet absorbance of methylparaben in both water and medium before and after autoclaving. No change was observed in the ultraviolet absorption spectrum. Solutions of Tween 80 were titrated with sodium hydroxide solution before and after autoclaving with no increase in the amount of sodium hydroxide required for neutralization to a phenolphthalein end point. It was thus assumed that the preservative and the Tween were stable to autoclaving.

Influence of Culture Medium on Interaction of Paraben with Tween.—To determine any influence which the culture medium might have on the binding of the preservative by the Tween 80, comparative dialysis studies were carried out in water and in the culture medium according to the procedure described in an earlier publication (1).

Studies on Aerobacter Aerogenes.—Growth studies were carried out in Pyrex test tubes, 25 x 150 mm. Tubes, each containing 20 ml. of medium with the desired concentration of Tween and paraben, were inoculated with a loopful of a twenty-four-hour culture of the bacteria grown in the synthetic medium. These tubes were then maintained at a temperature of 30°. Growth was followed by determination of turbidity at varying time intervals, using a Klett-Summerson photoelectric colorimeter with a No. 42 blue filter. Observations were made on two tubes at each time interval.

In addition, tubes containing medium with 0, 2, 4, and 6% Tween 80 and a series of concentrations differing by 0.005% in the plain medium and 0.02% in the Tween-containing medium, were prepared in duplicate and stored at 30° for daily gross visual observation over a period of one month. Inoculations of control and Tween-containing samples were made at the same time to avoid any variation in the inoculum.

Studies on Aspergillus niger.—The procedure for the inoculation of tubes with *Aspergillus niger* was identical to that for *Aerobacter aerogenes* with the exception that the inoculum consisted of a loopful of a spore suspension from a one-week culture of *Aspergillus niger* in the synthetic medium. Growth was observed visually as positive or negative in duplicate samples of the medium containing 0 and 7% Tween 80 and a series of paraben concentrations differing by 0.005% in the plain medium, and 0.02% in the Tween-containing medium.

Additional studies were carried out in which

[3] Methyl Parasept, purified, Heyden Newport Chemical Corp., New York.
[4] ATCC No. 8308.
[5] ATCC No. 10254.

the organism was separated from the Tween by a nylon membrane. Tween, paraben, and 20 ml. of medium were placed inside nylon sacks (1) which permitted free passage of the paraben, but which were impermeable to the Tween, the microorganism, and probably to any esterase produced by the microorganism. These bags were then placed in ground-glass-stoppered bottles containing 20 ml. of medium, and the entire assembly was sterilized by intermittent sterilization to avoid damage to the nylon membrane. The bottles were then agitated for fifteen hours at a temperature of 30°. This agitation permitted equilibrium to be established between the paraben and the macromolecule. The liquid outside the bag was then inoculated and the bottles were maintained at 30° for observation of growth.

RESULTS AND DISCUSSION

If the failure of normal inhibitory concentrations of the parabens in the presence of Tween 80 can be attributed primarily to the association between preservative and surfactant, and if the inhibition of microbial growth in absence of Tween 80 can be obtained by a paraben solution of a thermodynamic activity corresponding to less than saturation, it would follow that in the presence of any concentration of Tween 80 there must be some concentration of paraben less than saturation which will prevent microbial growth.

In any aqueous system containing Tween 80 and methylparaben, the total paraben present will consist of paraben bound to Tween and the free or unbound paraben.

$$\text{Paraben} + \text{Tween} \rightleftharpoons \text{Paraben-Tween}$$

It has been shown previously that for any given Tween 80 concentration the ratio of total to free methylparaben is a constant, r (1). If the preservative activity of the paraben can be assumed to be due to the unbound or available form, then it should be possible to calculate required preservative concentrations for any Tween 80 solution by multiplying the required concentration of free paraben by the appropriate r value. For these calculations it was assumed that the minimum inhibitory concentration of paraben determined in the absence of Tween corresponds to the concentration of free paraben required. Thus, for any given concentration of Tween: (Total paraben)/(Free paraben) × Required free paraben = Required preservative concentration.

Influence of Medium on Binding.—It was found that the interaction of the methylparaben with Tween 80 was not identical in a medium of distilled water and in the culture medium employed in these studies. As shown in Fig. 1, the paraben interacts with the Tween to a higher degree in the presence of the medium. The results for the binding in distilled water are in good agreement with those previously reported. It is therefore necessary to take into account the nature of the culture medium, even when it is a very simple synthetic medium, if the binding data are to be correlated with microbiological results.

Inhibitory Concentration for Aerobacter Aerogenes.—Inhibitory concentrations were obtained

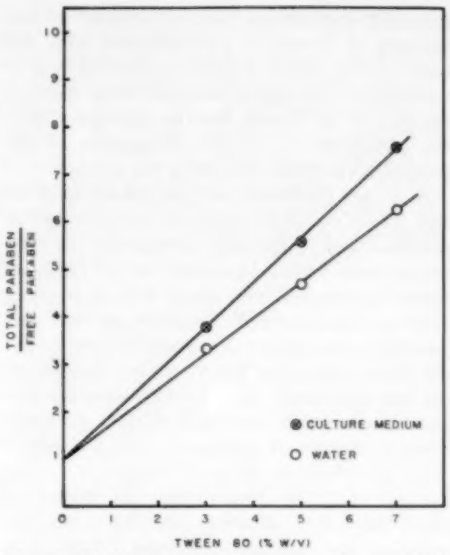

Fig. 1.—Binding of methylparaben by Tween 80 in water and in the culture medium, 30°.

for *Aerobacter aerogenes* in the plain medium and in media containing various concentrations of Tween 80. Higher concentrations were required in the presence of Tween 80, and these experimental concentrations were compared with the values which were predicted from a knowledge of the degree of interaction between paraben and Tween.

Figure 2 illustrates the influence of varying concentrations of methylparaben on the growth of *Aerobacter aerogenes* in a medium containing 5% Tween 80. The final inhibitory concentrations in these studies were determined by taking as the inhibitory concentration the range between the lowest paraben concentration not showing growth after one month, and the next lower paraben concentration.

Table I shows the minimum inhibitory concentration of methylparaben as a function of Tween 80 concentration.

TABLE I.—INHIBITORY CONCENTRATION OF METHYLPARABEN IN THE PRESENCE OF TWEEN 80, OBSERVED FOR ONE MONTH

Organism	Tween 80, %	Methylparaben, %
Aerobacter aerogenes	0	0.075–0.080
Aerobacter aerogenes	2	0.18–0.20
Aerobacter aerogenes	4	0.28–0.30
Aerobacter aerogenes	6	0.40–0.42
Aspergillus niger	0	0.045–0.050
Aspergillus niger	7	0.32–0.34

Inhibitory Concentration for Aspergillus niger.—The inhibitory concentration of methylparaben for *Aspergillus niger* in the plain medium was determined as 0.045–0.050%. The inhibitory concentration in the presence of 7% Tween 80 was 0.32–0.34% when the organism was in contact with the Tween, and when the organism was separated from

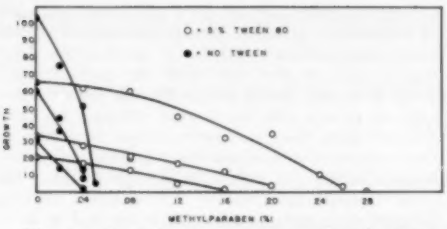

Fig. 2.—A typical growth curve for *Aerobacter aerogenes* in the presence of varying concentrations of methylparaben. The curves show increasing growth after one, two, and seven days, respectively, in plain medium and in medium containing 5% Tween 80. Growth is represented on an arbitrary scale based on turbidity measurements.

the Tween by a semipermeable membrane, but in contact with the paraben, the inhibitory concentration was found to be in the range 0.326–0.346%. The paraben bound by the nylon was taken into account in these calculations. The results suggest that the principal effect of the Tween is to bind the preservative, and any direct influence of Tween on the growth of the organism seems to be negligible so far as required preservative concentration is concerned.

It should be noted that in the studies utilizing the nylon membrane the absence of Tween outside the nylon membrane in concentrations detectable by analytical methods was confirmed, but it was not possible to prevent contamination by minute traces of the surfactant and the resulting decrease in surface tension in the external solution.

Correlation of Binding Data and Inhibitory Concentration.—Figure 3 shows a comparison of the experimental inhibitory concentrations of methylparaben in Tween 80 and the concentrations predicted from a knowledge of the inhibitory concentration in plain media and the ratio of total to free methylparaben at the given Tween concentration. The solid line in Fig. 3 represents a slope of 1, and would indicate agreement between experimental and predicted inhibitory concentrations. The predicted concentrations obtained by multiplying the required concentration in plain media by the r factor were slightly in excess of the experimental values. However, as illustrated in Fig. 4, the concentration of free paraben required to inhibit *Aerobacter aerogenes* in any Tween concentration studied was a constant, approximately 0.065%. This value is slightly less than the concentration of 0.075–0.080% required in plain media, and it is possible that this decrease in required concentration of free paraben may be due to the surface tension depression in Tween 80 solutions. Aoki, Kamata, Matsuzaki, and Nakatani (11) found that in extremely dilute solutions of Tween 20, where binding is probably negligible, lower concentrations of preservative were required than was the case in the absence of Tween. This effect would account for the deviation of the experimental values and predicted values illustrated in Fig. 3.

Although there is a dearth of data concerning the ratio of total to free preservative in the presence of nonionic surfactants, such information can often be obtained from solubility data (1). Thus the data of Chakravarty, Lach, and Blaug (14) for the solubility of methyl and propyl *p*-hydroxybenzoates

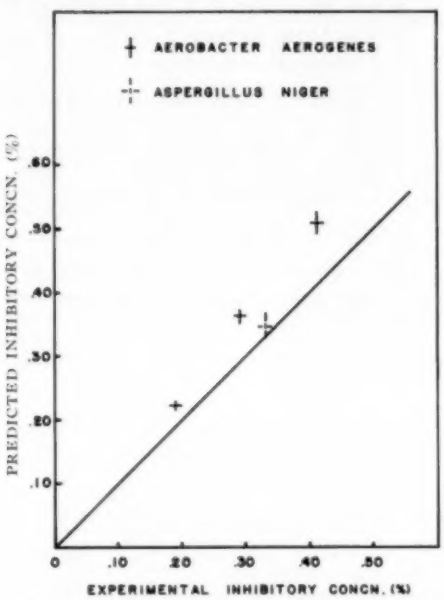

Fig. 3.—A comparison of predicted and experimental inhibitory concentrations of methylparaben in the presence of Tween 80.

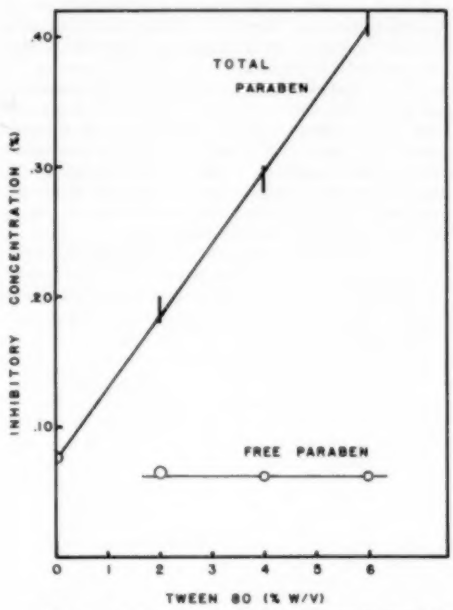

Fig. 4.—A comparison of the total methylparaben concentration and the free methylparaben concentration required to inhibit growth of *Aerobacter aerogenes* in the presence of Tween 80.

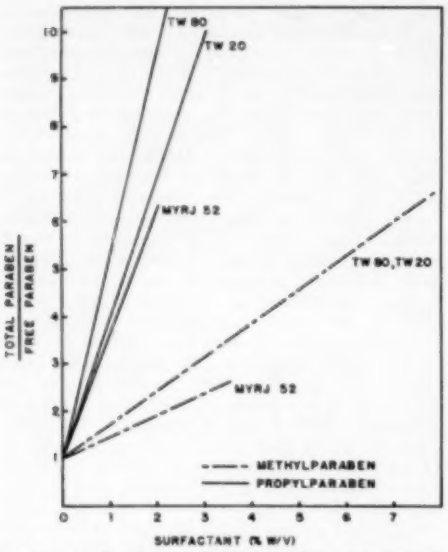

Fig. 5.—Binding of methylparaben and propylparaben by several representative surfactants of the polyoxyethylene type. Data for Tween 80 are from Patel and Kostenbauder (1); data for Myrj 52 were calculated from the solubility study of Chakravarty, Lach, and Blaug (14); and data for Tween 20 were calculated from antifungal concentrations reported by Aoki, *et al.* (9).

in Myrj 52[6] can be employed to calculate the ratio of total to free preservative as a function of the concentration of Myrj 52. These data are illustrated in Fig. 5 for comparison with the binding exhibited by Tween 80.

The ratios of total to free paraben in the presence of Tween 20, shown in Fig. 5, were estimated from inhibitory concentrations reported by Aoki, Kamata, Yoshioka, and Matsuzaki (9). These determinations were made in modified Sabouraud media, but the results seem to indicate that the binding of the parabens by Tween 20 would be quite similar to that of Tween 80 and Myrj 52.

It is significant that the interaction of propylparaben and other relatively hydrophobic esters with Tween is much greater than the interaction of the methyl ester. Thus, for a given concentration of surfactant, a much greater proportion of the total propylparaben will be in an inactive form than would be the case with the methyl ester. Aoki, *et al.* (9), noted that while the higher esters such as propyl and butyl were effective in lower concentration than the methyl ester in water, in the presence of surfactants this relationship is sometimes reversed. It should be possible to select the most suitable preservative for a given system, utilizing data such as those presented in Fig. 5.

[6] Myrj 52 is polyoxyl 40 stearate, Atlas Powder Co., Wilmington, Del.

SUMMARY

1. The preservative activity of *p*-hydroxybenzoic acid esters in the presence of nonionic surfactants is primarily a function of the concentration of unbound preservative.

2. The *p*-hydroxybenzoates can be employed as efficient preservatives in the presence of nonionic surfactants, but the proper concentrations must be employed. This study suggests that these concentrations can be estimated by multiplying the usual preservative concentration by the ratio of total to free paraben at any given surfactant concentration.

3. Hydrophobic esters such as propyl and butyl are usually effective in lower concentrations than the methyl ester when employed in aqueous systems, but in the presence of surfactants of the polyoxyethylene type this relationship might sometimes be reversed.

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Interaction of Preservatives With Macromolecules III*

Parahydroxybenzoic Acid Esters in the Presence of Some Hydrophilic Polymers

By GRACE M. MIYAWAKI†, N. K. PATEL‡, and H. B. KOSTENBAUDER

The magnitude of any intermolecular association occurring in aqueous solution between methyl- and propylparaben and several hydrophilic polymers was determined. Parabens were found to interact to some extent with polyethylene glycol 4000, methylcellulose, polyvinylpyrrolidone, and gelatin. There was no evidence for a significant degree of interaction between the parabens and carboxymethylcellulose or tragacanth. The significance of these results relative to preservation of solutions containing these polymers is considered.

PREVIOUS STUDIES in this series (1, 2) have been concerned with the rather significant interaction of preservatives with nonionic surfactants of the polyoxyethylene type and the importance of considering such interactions when determining proper preservative concentrations. Several articles published in recent years indicate that some thought has been given to interactions of preservatives with macromolecules such as tragacanth (3, 4), methylcellulose (3, 5), carboxymethylcellulose (3), Irish moss (3), and polyethylene glycols (6). The purpose of the present investigation was to determine the extent of any such interactions which might occur between *p*-hydroxybenzoic acid esters and some representative macromolecules, and thus obtain an evaluation of the relative importance of such binding in determining required preservative concentrations.

Studies were performed on polyethylene glycol 4000, methylcellulose, polyvinylpyrrolidone, gelatin, carboxymethylcellulose, and tragacanth, using solubility and equilibrium dialysis techniques to detect any possible association between the parabens and the macromolecules.

EXPERIMENTAL

Reagents.—Recrystallized methyl *p*-hydroxybenzoate,[1] m. p. 128–128.5°; recrystallized propyl *p*-hydroxybenzoate,[2] m. p. 96–98°; polyethylene glycol 4000;[3] methylcellulose 15 c. p. s.;[4] carboxymethylcellulose, low viscosity;[5] tragacanth;[6] gelatin, derived from an acid-treated precursor;[7] and polyvinylpyrrolidone.[8] The concentrations of all macromolecular dispersions are expressed on a w/v basis and have been corrected for moisture content as determined by drying a sample of the material to constant weight.

Equilibrium Dialysis Method.—The equilibrium dialysis technique employed in the present study is essentially the same in principle as that used by Klotz, Walker, and Pivan (7) and Karush and Sonenberg (8) in investigating the binding action of proteins, and by Higuchi and co-workers (9, 10, 11) in their studies of the interaction between some macromolecules and pharmaceuticals. The experimental technique was identical to that used by Patel and Kostenbauder (1) in studying the interaction of parabens with Tween 80,[9] with the exception that the membrane employed in these studies was cellulose dialyzer tubing.[10]

The dialysis method was employed for the study of methylcellulose, polyvinylpyrrolidone, gelatin, carboxymethylcellulose, and tragacanth.[11] Twenty-five ml. of the polymer solution under study was placed in each cellophane bag. Each bag was then tightly tied and placed in a wide-mouth glass-stoppered bottle containing 25, 50, or 75 ml. of an aqueous paraben solution. The capped bottles were then agitated for fifteen hours at constant temperature. For the more viscous tragacanth dispersions a longer period of equilibration was required and the above method was modified in that the paraben was dissolved in the tragacanth dispersion and the bottles containing these samples were agitated for several days until constant assay results were obtained for the external solution. The method of assay and the treatment of data have been described previously (1).

The volume of the solution outside the membrane was measured, after equilibration, to permit correction of the polymer concentration in the event a volume change had occurred. Important volume changes were noted only in the carboxymethylcellulose solutions.

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This work was supported in part by a research grant from Heyden Newport Chemical Corp., New York.
† Chas. Pfizer Fellow in Hospital Pharmacy, 1957–1958.
‡ Present address: School of Pharmacy, University of Maryland, Baltimore, Md.
[1] Methyl Parasept, purified, supplied through the courtesy of Heyden Newport Chemical Corp., New York.
[2] Propyl Parasept, purified, supplied through the courtesy of Heyden Newport Chemical Corp., New York.
[3] "Carbowax" 4000, supplied through the courtesy of Union Carbide Chemicals Co., New York.
[4] Methocel 15 c. p. s., supplied through the courtesy of Dow Chemical Co., Midland, Mich.
[5] CMC, 70 premium low, supplied through the courtesy of Hercules Powder Co., Wilmington, Del.
[6] S. B. Penick & Co., New York.
[7] Pharmagel A, Pharmagel Corp., New York.
[8] Plasdone, supplied through the courtesy of Antara Chemicals, New York.
[9] Tween 80 is polyoxyethylene (20) sorbitan monooleate, Atlas Powder Co., Wilmington, Del.
[10] A. H. Thomas, Philadelphia, Pa., average pore diameter 48 Ångström.
[11] Tragacanth contains a small fraction of dialyzable material; but any error introduced by neglecting this fraction is probably insignificant, particularly in view of the nature of the data obtained.

Solubility Method.—The solubility of methyl and propyl esters of *p*-hydroxybenzoic acid was determined in the presence of varying concentrations of polyethylene glycol 4000. Excess quantities of paraben (0.25 Gm. of the propyl ester, 1.25 Gm. of the methyl ester) were placed in 125-ml. glass-stoppered bottles containing 50 ml. of polyethylene glycol 4000 solution. The bottles were agitated for twenty-four hours at 30°. The method of assay and treatment of data have been described previously (1).

RESULTS AND DISCUSSION

Polyethylene Glycol 4000.—Lach, Ravel, and Blaug (6) determined the solubility of methyl and propyl *p*-hydroxybenzoates in aqueous solutions containing up to 2% PEG 4000 and PEG 6000. In the present study the solubility determinations were extended to 10% w/v PEG 4000, as illustrated in Fig. 1. In Fig. 2 the solubility data have been plotted to show the ratio of total to free paraben as a function of PEG 4000 concentration. As illustrated in a previous study (2), the ratio, *r*, shown in Fig. 2, can be used as a first approximation in determining any increased quantity of preservative which might be required in the presence of the PEG. For example, if it can be assumed that any interference with the activity of the preservative is due to binding of the macromolecule, and the macromolecule itself exhibits no antimicrobial activity, then in a solution of 5% PEG 4000 it might be expected that the concentration of methylparaben employed should be approximately 1.6 times the concentration which would be employed in the absence of the PEG. While this approach is undoubtedly an oversimplification of actual conditions, it nevertheless provides some indication of the quantity of preservative which might be required.

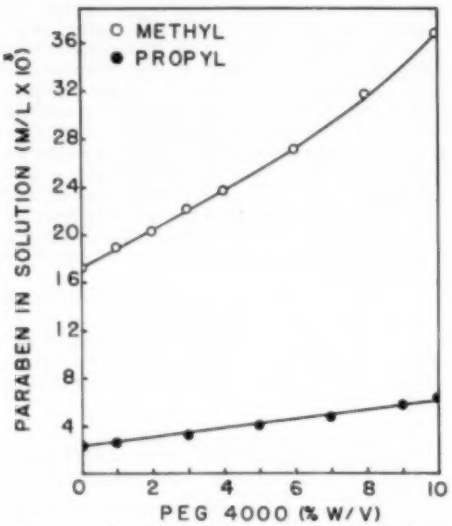

Fig. 1.—Solubility of methyl and propyl *p*-hydroxybenzoate in aqueous solutions of polyethylene glycol 4000 at 30°.

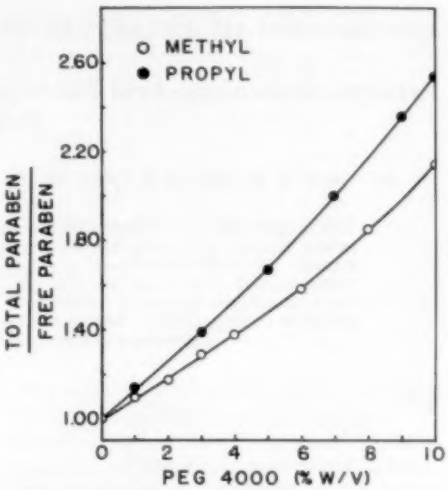

Fig. 2.—Ratio, *r*, of total paraben to free paraben as a function of polyethylene glycol 4000 concentration at 30°.

The binding of parabens by PEG is of minor importance in comparison with the binding exhibited by polyoxyethylene surfactants (1, 2), and results reported by deNavarre and Bailey (12) suggest that in practice there is little or no interference with the preservative activity of methylparaben in the presence of 2% PEG 4000. This result is not surprising, since preservatives are usually employed in concentrations well in excess of the minimum inhibitory concentration; thus, binding a portion of the preservative might not reduce the effective concentration to a level which would permit microbial growth.

Figure 2 indicates that the propyl ester is bound to a greater extent than the methyl ester, but the difference in binding affinity is considerably less than was observed for these two compounds in solutions of polyoxyethylene surfactants (2).

Methylcellulose.—Tillman and Kuramoto (5) studied the solubility of several parabens in the presence of methylcellulose and obtained evidence which would suggest the existence of both soluble and insoluble complexes between the parabens and this polymer. It is possible that the phenolic group of the paraben might interact with the ether groups of the methylcellulose in a manner analogous to the interaction with PEG.

The dialysis method was employed in the present study to obtain an estimate of the degree of interaction in the concentration range in which the parabens might normally be employed. Figure 3 illustrates the ratio of total to free paraben as a function of methylcellulose concentration. While there is definite evidence that an interaction does occur, the ratio of total to free paraben indicates that in the concentrations in which methylcellulose might normally be employed it is unlikely that sufficient paraben would be bound to the macromolecule to necessitate the addition of supplementary quantities of paraben.

Polyvinylpyrrolidone.—The interaction of methyl and propyl *p*-hydroxybenzoates with PVP was investigated by means of the dialysis technique. The

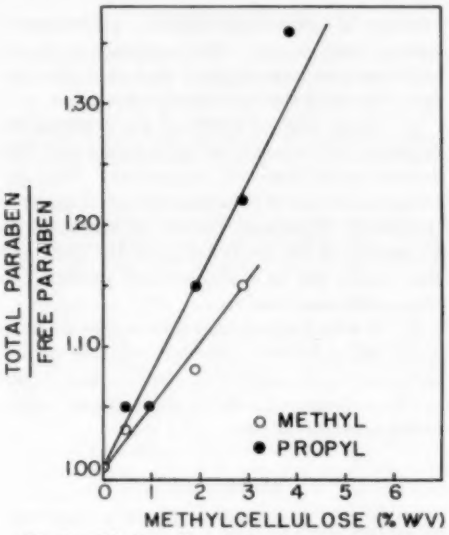

Fig. 3.—Binding of methyl and propyl *p*-hydroxybenzoate by methylcellulose at 30°. Total methylparaben in solution 6.41×10^{-3} *M*; total propylparaben in solution 1.23×10^{-4} *M*.

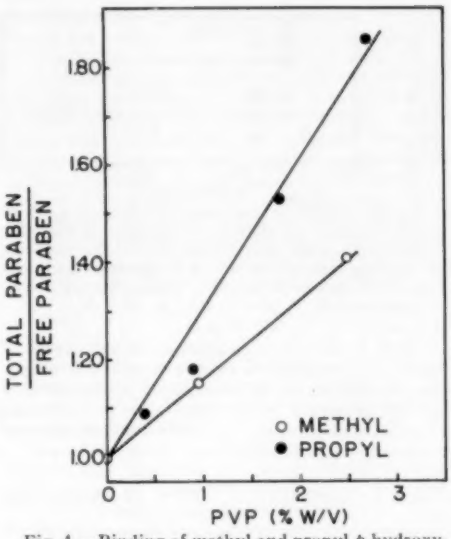

Fig. 4.—Binding of methyl and propyl *p*-hydroxybenzoate by polyvinylpyrrolidone. Data for methylparaben obtained at 27°, and data for propylparaben at 30°. Total methylparaben in solution 6.12×10^{-3} *M*; total propylparaben in solution 1.23×10^{-3} *M*.

ratio of total to free paraben as a function of PVP concentration is illustrated in Fig. 4.

PVP contains an amido group; and it has previously been demonstrated that compounds such as phenols, which are capable of acting as proton donors, tend to form complexes with amides (9, 13). Of the polymers included in this study PVP exhibited maximum binding of the parabens.

Gelatin.—The results of a dialysis study of methyl and propylparaben in the presence of gelatin provided evidence, as indicated in Fig. 5, for some degree of interaction. These results were obtained in unbuffered solutions at a pH of 4.3–4.4, using gelatin derived from an acid-treated precursor. The interaction of the paraben with the gelatin can probably be attributed to the presence of the amide-type groups in the gelatin.

The degree of binding observed in the presence of gelatin appears to be of relatively minor importance in determining required preservative concentrations.

Carboxymethylcellulose.—The possible interaction of CMC with methylparaben was investigated by means of the equilibrium dialysis method and the results are presented in Fig. 6. Extreme volume changes occurred in these solutions due to the osmotic activity of the CMC. To prevent these changes when attempting to investigate more concentrated CMC solutions it was necessary to add sodium chloride as a swamping electrolyte. In no case was there any detectable evidence of association between the paraben and the CMC.

Tragacanth.—Dialysis data for methylparaben in the presence of tragacanth are presented in Fig. 6. The results of these studies indicate that there is no significant binding of the preservative by the tragacanth. If increased quantities of the parabens should be required for effective preservation of tragacanth dispersions this increase must be attributed to some factor other than adsorption of the preservative. It is probable that tragacanth dispersions provide a more favorable medium for the growth of microorganisms; and, if so, it might be expected that the necessity for increased preservative concentrations would not be limited to any specific preservative.

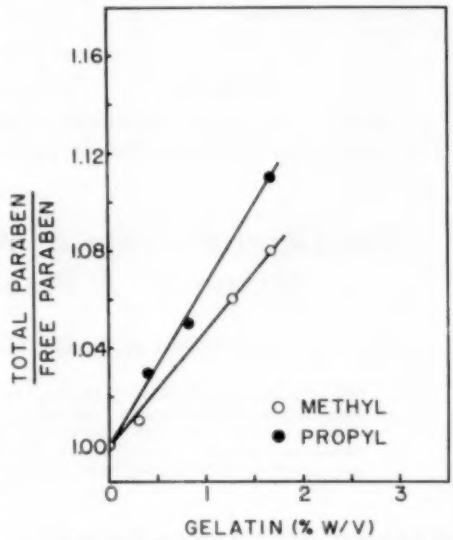

Fig. 5.—Binding of methyl and propyl *p*-hydroxybenzoate by gelatin at 30°. Total methylparaben in solution 9.80×10^{-3} *M*; total propylparaben in solution 1.85×10^{-3} *M*.

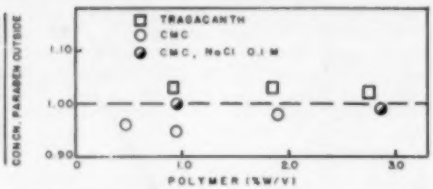

Fig. 6.—Ratio of the concentration of methylparaben inside the dialysis membrane to the concentration outside the membrane, in the presence of varying concentrations of tragacanth and carboxymethylcellulose at 30°. Methylparaben concentration 6.60×10^{-3} to 9.88×10^{-3} *M*.

Table I presents a comparison of the degree of binding of parabens in the presence of 2% dispersions of the macromolecules studied. Although data are not presented for propylparaben in CMC and tragacanth dispersions, the results would be expected to parallel those obtained with the methyl ester.

TABLE I.—COMPARATIVE BINDING OF PARABENS BY 2% POLYMER SOLUTIONS

Macromolecule 2% w/v	Total Methyl paraben Free, %	Total Propyl-paraben Free, %
Polyvinylpyrrolidone	78	64
Polyethylene glycol 4000	84	81
Methylcellulose	91	87
Gelatin	92	89
Carboxymethylcellulose	100	..
Tragacanth	100	..

SUMMARY

1. Methyl and propyl *p*-hydroxybenzoate were found to interact to some extent with polyethylene glycol, methylcellulose, polyvinylpyrrolidone, and gelatin. The magnitude of the association is considerably less than that observed previously with several nonionic surfactants.

2. There was no evidence for a significant degree of interaction of methylparaben with carboxymethylcellulose or tragacanth. Any increased quantity of paraben required to preserve tragacanth dispersions cannot be attributed to adsorption of the preservative by the gum, and thus should not be a phenomenon restricted to any specific preservative.

3. It would appear that for concentrations in which the polymers under investigation are usually employed, binding of the parabens would not be sufficient to prohibit their efficient application as preservatives.

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The Determination of Acetophenetidin in Tablet Mixtures: A Potentiometric Method*

By JOSEPH G. BALDINUS and IRVIN ROTHBERG

When acetophenetidin is hydrolyzed with acid, the resulting *p*-phenetidin can be titrated potentiometrically with sodium nitrite if potassium bromide is used as a catalyst to speed the diazotization reaction. This provides the basis for a simple, rapid, and accurate procedure for determining acetophenetidin. Aspirin, caffeine, salicylic acid, codeine, amphetamine, and amobarbital do not interfere with the titration.

RECENTLY, Falex (1) reported that *p*-phenetidin could be titrated with sodium nitrite using starch-iodide paper as an indicator. This would be an attractive procedure for acetophenetidin, except for a number of disadvantages: the starch-iodide paper is insensitive to dilute solutions of sodium nitrite, the temperature must be controlled, colored solutions give trouble, the reaction with starch-iodide paper requires considerable time, and, finally, the use of an external indicator is simply not conducive to accuracy and precision.

In the work presented below, the diazotization reaction was speeded so that the titration of the *p*-phenetidin could be followed potentiometrically. The resulting procedure has proved considerably less troublesome than the existing methods for determining acetophenetidin (2–10).

* Received October 7, 1958, from the Smith Kline and French Laboratories, Philadelphia 1, Pa.

EXPERIMENTAL

Reagents.—A 0.05 *N* sodium nitrite solution which was prepared by dissolving approximately 3.5 Gm. sodium nitrite in one liter of distilled water; sulfanilamide, U. S. P. reference standard, which was used to standardize the nitrite solution; potassium bromide crystals; and a 10% aqueous solution of HCl.

Apparatus.—Either a Photovolt model 110 or a Beckman model G pH meter; a platinum electrode which was used *versus* a calomel electrode; a timer, preferably one with a second hand; and a 25-ml. buret.

Procedure.—The tablets were ground to a fine powder and an amount of sample that contained about 160–180 mg. of acetophenetidin was refluxed for one to one and one-half hours with 50 ml. of 10% aqueous HCl. The resulting solution was transferred quantitatively to a 250-ml. beaker with the aid of 50 ml. 10% aqueous HCl, 5 Gm. of potassium bromide was added, and the solution was then titrated potentiometrically with sodium nitrite, using a magnetic stirrer for agitation. At the beginning of the titration, the titrant was added rapidly, but near the end point, increments of 0.1 ml. were added. Since the potential drifted during the titration, the readings were recorded exactly one minute after each addition of titrant. The inflection point was calculated by the method of Kolthoff and Laitinen (11).

A blank titration was also run on the sample prior to hydrolysis with acid. On all our samples, the blanks were negligible. Ordinarily, the blank would be nil unless *p*-phenetidin had somehow formed in the tablets. This conceivably could happen with age or under unusual storage conditions. For control work, the blank determination could probably be omitted altogether. After subtraction of the blank, each ml. of 0.05 *N* sodium nitrite consumed was equivalent to 8.955 mg. acetophenetidin.

In standardizing the sodium nitrite with sulfanilamide, it was not necessary to use potassium bromide. However, the potentials were still recorded as above, one minute after the addition of each increment of titrant.

DISCUSSION AND RESULTS

Without the use of potassium bromide, a potentiometric end point could not be obtained when *p*-phenetidin was titrated with sodium nitrite. Concentrations of potassium bromide ranging from 1–10% were tried, and the optimum concentration was found to be about 5%. Below concentrations of 4%, the size of the end point seemed to diminish; above 5% no improvement was noted.

Iodide has been reported (12) to be an even more effective catalyst for the diazotization of aromatic amines than bromide. While this may be true at very low acidities, in our titrations the iodide was simply oxidized by the sodium nitrite.

The dead stop method (13) using polarized platinum electrodes was also tried. With potassium bromide, the end point could be detected; however, it was not nearly so pronounced or so reproducible, as was the potentiometric end point.

Caffeine, aspirin, salicylic acid, and the common tablet excipients, such as starch, talc, magnesium stearate, calcium sulfate, and guar gum were checked for possible interference in the titration; however, no interference was found. Codeine, amphetamine, and amobarbital, also, did not affect the titration.

In the blank titration, the acetophenetidin comes in contact with a 10% acid solution and might be expected to hydrolyze slightly. Pure acetophenetidin, however, gave a negligible blank even after thirty minutes stirring in the acidic solution.

RESULTS

When pure acetophenetidin was analyzed by the above procedure, theoretical recoveries were obtained. Results obtained on various tablet mixtures are reported in Table I.

TABLE I.—ANALYSIS OF ACETOPHENETIDIN IN VARIOUS TABLETS

Sample	Label Declaration, gr./tablet	Found, gr./tablet
1[a]	5.00	4.94
2	5.00	5.02
3	5.00	5.00
4	5.00	4.95
5	5.00	4.97
6	5.00	4.94
7	5.00	5.00
8	5.00	4.96
9	5.00	4.95
10	5.00	4.98
11	5.00	5.02
12	5.00	5.02
13[b]	2.50	2.50
14[b]	2.50	2.52
15[c]	2.50	2.48
16[c]	2.50	2.50

[a] Samples 1–12 inclusive are APC tablets.
[b] These tablets contain codeine sulfate, amphetamine sulfate, and aspirin in addition to the acetophenetidin.
[c] Amphetamine sulfate, amobarbital, and aspirin are present in these samples.

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Substituted α-Benzylphenethylamines*

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A series of amines related to α-benzylphenethylamine was prepared. The preparation of the penicillin G salts of these amines was attempted. The most interesting compounds reported are the penicillin G salts of α-benzyl-N-(3-phenylpropyl)-phenethylamine and α-benzyl-N-(p-butoxybenzyl)-phenethylamine.

MUCH EFFORT in the pharmaceutical industry has been expended to obtain formulations of penicillin G derivatives which would be stable for extended periods and yet yield high blood levels upon oral administration. The most notable of the insoluble amine-penicillin salts prepared for this purpose has been benzathine penicillin G (1).

α,N-Dibenzylphenethylamine (Table I, 3)[1] was found to form a crystalline salt with penicillin G. A series of related amines was synthesized in order to select the most desirable penicillin salt for further study. *p*-Substitution of the N-benzyl moiety by various alkoxy, alkyl, or halogen groups gave crystalline penicillin salts of solubilities[2] ranging from 0.05 to 0.009%. Substitution of other groups such as nitro or acetamido at this position gave amorphous salts. *o*- or *m*-Substitution gave uniformly negative results. Replacement of N-benzyl by N-cyclohexylmethyl, N-hexadecyl, or N-furyl-methyl moieties gave bases which would not form crystalline salts. Methylation of I, 3 by the Clark-Eschweiler reaction destroyed its ability to form crystalline salts.

Crystalline penicillin salts of low solubility were obtained from amines prepared by replacement of the N-benzyl group by phenyl substituted straight chain alkanes. The most interesting member of this group was the α-benzyl-N-(3-phenylpropyl)-phenethylamine penicillin salt (Table IV, 2). The amine (Table I, 5) was prepared most readily by the reductive alkylation of α-benzylphenethyl amine by hydrocinnamaldehyde. When cinnamaldehyde was used in the alkylation, lower yields resulted due to the formation of considerable amounts of a by-product, N-(α-benzylphenethyl)-cinnamylamine. Lower yields were the result of alkylation by phenylpropyl bromide or chloride.

An alternate method of synthesis was developed, consisting of Meerwein-Ponndorf-Verley reduction of dibenzyl ketone to the carbinol which was reacted with hydrogen bromide to give α-benzylphenethyl bromide. Although the bromide could not be purified easily, the crude compound was condensed with appropriate amines to obtain compounds not obtained readily otherwise.

The conversion of dibenzalacetone to 1-phenethyl-3-phenyl-propylamine via the reduction of the oxime has been reported (2), but the procedure proved to be very tedious in our hands. The ketone was reduced catalytically (3) with 5% palladium-on-charcoal until two molar equivalents of hydrogen had been absorbed. The Leuckart reaction using formamide gave a 76% yield of the desired amine. 5-Phenyl-1-(4-phenylbutyl)-pentylamine was prepared in similar fashion from 1,9-diphenyl-1,3,6,8-nonatetraen-5-one via 1,9-diphenyl-5-nonanone. None of a series of alkylated derivatives of these amines formed crystalline penicillin salts.

The dry distillation of the calcium salt of α-naphthylacetic acid gave 1,3-di-(α-naphthyl)-2-propanone. A Leukart amination and subsequent alkylation gave the dinaphthyl analog of I, 3 which formed an amorphous penicillin salt.

Hydrogenation of α-benzylphenethylamine with ruthenium dioxide catalyst at medium pressures and elevated temperatures resulted in a good yield of α-cyclohexylmethylcyclohexylethylamine. Alkylated derivatives of this amine were only partially successful in forming penicillin salts.

The pKa values of representative members of the series were determined by electrometric titration, usually in 5% *n*-propanol (4) in an attempt to correlate a physical property of the amines with the character of their penicillin salts. Although the variation in basicity with structure was largely that expected, no correlation in these values with the crystallinity of the penicillin salts was apparent. In general, alternations of the parent skeleton of I, 3 which destroyed the symmetry of the amine resulted in loss of the crystallinity of its penicillin salt.

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[1] Compounds are designated by Table No. and entry No.

[2] Solubilities were determined most conveniently by a weight differential method developed by Mr. J. A. Sultzaberger of this laboratory. A method based on ultraviolet assay of the filtrate after agitation of a suspension of the salt gave erratic results.

TABLE I.—α-BENZYL-PHENETHYLAMINES

$$C_6H_5—CH_2—\underset{\displaystyle NRR'}{\underset{|}{CH}}—CH_2C_6H_5$$

No.	R	R′	M. P., °C.	Yield, %	pK′a	Prepn.	Formula	Analyses, %: Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
1	CH_3	CH_3	223	83	..	[a]	$C_{17}H_{21}N.HCl$	79.03	74.14	8.09	7.87
2	H	$C_{16}H_{33}$	107	54	5.4	B	$C_{31}H_{58}N.HCl$	78.85	78.41	10.68	10.87
3	H	$CH_2C_6H_5$	246	94	6.5	A, C	$C_{23}H_{24}N.HCl$	78.20	77.61	7.16	7.39
4	H	$C_2H_4C_6H_5$	188	64	6.7	A, B	$C_{24}H_{26}N.HBr$	69.70	69.61	6.61	6.72
5	H	$C_3H_6C_6H_5$	193	79	7.2	A, B	$C_{25}H_{27}N.HCl$	78.77	78.92	7.71	7.55
6	H	$CH_2—CH(C_5H_{11})—CH_2C_6H_5$	146	46	..	A[b]	$C_{19}H_{25}N.HCl$	79.87	80.30	8.78	8.58
7	H	CH_2-C_6H_{11}	247	65	6.95	A[b]	$C_{23}H_{29}N.HCl$	76.81	76.68	8.79	9.01
8	CH_3	$CH_2C_6H_5$	160	71	..	[a]	$C_{24}H_{28}N.HCl$	78.50	78.26	7.45	7.20
9	H	$CH_2C_6H_4$-p-CH_3	242	52	..	B	$C_{24}H_{28}N.HCl$	78.50	78.53	7.45	7.57
10	H	$CH_2C_6H_4$-p-$CH(CH_3)_2$	211	50	6.55	A	$C_{26}H_{32}N.HCl$	79.02	79.02	7.94	8.20
11	H	$CH_2C_6H_4$-p-OCH_3	225	81	..	A	$C_{23}H_{26}NO.HCl$	75.08	75.19	7.12	6.82
12	H	$CH_2C_6H_4$-p-OC_2H_5	222	85	..	A	$C_{24}H_{27}NO.HCl$	75.47	75.93	7.39	7.49
13	H	$CH_2C_6H_4$-m-OC_2H_5	139	67	6.4	A	$C_{24}H_{27}NO.HCl$	75.47	75.63	7.39	7.62
14	H	$CH_2C_6H_4$-o-OC_2H_5	192	83	6.7	A	$C_{24}H_{27}NO.HCl$	75.47	76.10	7.39	7.58
15	H	$CH_2C_6H_4$-p-OC_3H_7	209	84	..	A	$C_{25}H_{29}NO.HCl$	75.83	75.74	7.64	7.60
16	H	$CH_2C_6H_4$-p-$OCH(CH_3)_2$	232	80	..	A	$C_{25}H_{29}NO.HCl$	75.83	76.24	7.64	7.73
17	H	$CH_2C_6H_4$-p-OC_4H_9	203	82	6.65	A	$C_{26}H_{31}NO.HCl$	76.16	76.34	7.87	7.89
18	H	$CH_2C_6H_4$-p-$OCH(CH_3)C_2H_5$	229	81	..	A	$C_{26}H_{31}NO.HCl$	76.16	76.14	7.87	8.12
19	H	$CH_2C_6H_4$-p-OC_5H_{11}	199	82	..	A	$C_{27}H_{33}NO.HCl$	76.48	76.49	8.08	8.29
20	H	$CH_2C_6H_4$-o-OC_5H_{11}	139	39	..	A	$C_{27}H_{33}NO.HCl$	76.48	76.30	8.08	8.30
21	H	$CH_2C_6H_4$-p-$OC_3H_6CH(CH_3)_2$	220	82	..	A	$C_{27}H_{33}NO.HCl$	76.48	76.77	8.08	8.16
22	H	$CH_2C_6H_4$-p-$OC_{16}H_{33}$	173	80	..	A	$C_{37}H_{63}NO.HCl$	77.78	77.80	8.98	8.93
23	H	$CH_2C_6H_4$-p-$OCH_2C_6H_5$	225	83	6.65	A	$C_{29}H_{29}NO.HCl$	78.44	78.62	6.81	6.89
24	H	$CH_2C_6H_4$-p-OC_2H_4Cl	207	85	..	A	$C_{25}H_{26}ClNO.HCl$	69.76	69.69	6.79	6.89
25	H	$CH_2C_6H_4$-p-Cl	257	14	6.1	A	$C_{23}H_{23}ClN.HCl$	70.96	71.50	6.23	6.50
26	H	$CH_2C_6H_4$-p-NO_2[c]	268	27	7.7	B	$C_{23}H_{23}N_2O_2HCl$	69.01	69.21	6.06	5.95
27	H	$CH_2C_6H_4$-p-$NHCOCH_3$	271	94	..	A	$C_{26}H_{28}N_2O.HCl$	72.98	72.89	7.05	6.82
28	H	$CH_2C_6H_4$-p-$N(C_2H_5)_2$	199	48	..	A	$C_{28}H_{32}N_2.2HCl.-\frac{1}{4}H_2O$[d]	68.03	68.08	7.80	8.09
29	H	$CH_2C_6H_2$-m-OCH_3-p-OH	205	78	..	A	$C_{23}H_{27}NO_2HCl$	72.44	72.60	7.09	7.23
30	H	CH_2-α-C_4H_3O	189	80	..	A	$C_{26}H_{31}NO.HCl$	73.27	72.89	6.76	7.09
31	H	H	209	..	8.0	..	$C_{15}H_{17}N.HCl$	72.71	72.39	7.32	7.14

[a] These compounds were prepared by Clarke Eschweiler methylation.
[b] The readily available unsaturated aldehydes were used in the reductive alkylation.
[c] Catalytic reduction of the *p*-nitro compound gave a solid which could not be purified.
[d] This compound, after drying *in vacuo*, was observed to be hygroscopic.

On the basis of low solubility in water, ease of crystallization, and preparation as well as commerical availability, the penicillin salts of α - benzyl - N - (*p* - butoxybenzyl) - phenethyl amine (Table IV, 9) and α-benzyl-N-(3-phenylpropyl)-phenethylamine (Table IV, 2) were chosen for further study. These salts produced blood levels considerably higher than those obtained with benzethine penicillin G in dogs following oral administration. Both of these compounds caused irritation when administered by injection. Many of the amines prepared proved to be potent local anesthetics.

EXPERIMENTAL[1]

α-Benzylphenethylamine was prepared by the method of Koelsch (5). Alkoxybenzaldehydes were prepared by the method of Stoermer and Wodarg as well as Weygand and Gabler (6). Several aldehydes not previously reported are *p*-decyloxybenzaldehyde (b. p. 173–176° at 1 mm.), *p*-chloropropoxybenzaldehyde (b. p. 110–115° at 1 mm.), *m*-amoxybenzaldehyde (b. p. 109° at 0.5 mm.) and *sec*-butoxybenzaldehyde (b. p. 135–137° at 6 mm.).

α-Benzylphenethylamines.—*Method A.*—An equimolar mixture of amine and aldehyde in ethanol was heated at reflux for one hour. Appropriate catalyst, usually platinum oxide or 5% palladium-on-charcoal, was added and the mixture hydrogenated at three atmospheres at room temperature to completion. After filtration and evaporation *in vacuo*, the residue was dissolved in ether. The ether extract was dried over potassium hydroxide, filtered, and saturated with dry hydrogen chloride gas. The crystalline product was usually recrystallized from aqueous ethanol.

Method B.—The primary amine was heated with a slight excess of appropriate alkyl halide with or without sodium carbonate for ten to twenty hours. The mixture was then filtered and the product isolated either as the hydrochloride or the free base.

Method C.—The reduction of dibenzyl ketone by magnesium-methanol (7) or catalytic hydrogenation gave poor returns of dibenzyl carbinol. A 70% yield of the alcohol was obtained using aluminum isopropoxide in the Meerwein-Ponndorf-Verley reaction.

[1] All melting points and boiling points are uncorrected.

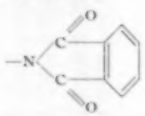
TABLE II.—α-PHENYLALKYL-PHENYLALKYLAMINES

$$C_6H_5(CH_2)_X—\underset{\displaystyle NHR}{\underset{|}{CH}}—(CH_2)_YC_6H_5$$

No.	R	X	Y	M. P., °C.	Yield, %	pK'a	Prepn.	Formula	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
1	$CH_2C_6H_5$	2	0	210	81	6.8	A	$C_{23}H_{24}N.HCl$	78.20	78.29	7.16	7.25
2	H	2	2	160	83	8.8	[a]	$C_{17}H_{21}N.HCl$	74.02	73.92	8.04	8.02
3	$CH_2C_6H_5$	2	2	118	79	7.5	A	$C_{24}H_{27}N.HCl$	78.77	78.55	7.44	7.80
4	$C_6H_5C_6H_5$	2	2	159	91	..	B	$C_{28}H_{29}N.HCl$	79.02	78.87	7.96	8.11
5	$C_6H_5C_6H_5$	2	2	88	75	..	A	$C_{16}H_{21}N.HCl.{}^1/_2H_2O$	77.48	77.35	8.25	8.25[b]
6	$CH_2C_6H_4$-*p*-OCH_3	2	2	147	92	..	A	$C_{25}H_{28}NO.HCl$	75.83	75.85	7.64	7.74
7	H	4	4	[c]	31	..	[d]	$C_{21}H_{29}N$	85.36	84.70	9.89	9.89
8	$CH_2C_6H_4$-*p*-OCH_3	4	4	98	63	..	A	$C_{33}H_{37}NO.HCl$	77.04	76.81	8.47	8.80[e]

[a] Previously reported by Stuhmer and Kaupmann (2). [b] Calcd.: N, 3.47. Found: N, 3.30. [c] B. p. 177–178° at 0.7 mm. [d] See Experimental. [e] Calcd.: N, 3.09. Found: N, 3.19.

TABLE III.—MISCELLANEOUS RELATED AMINES

No.	Structure	M. P.	Yield	pK'a	Prepn.	Formula	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
1	$(C_6H_{11}CH_2)_2CH$-$NHCH_2C_6H_5$	208	76	7.7	A	$C_{32}H_{43}N.HCl$	75.50	75.45	10.37	10.20
2	$(C_6H_{11}CH_2)_2CHNHC_6H_4C_6H_5$	120	40	7.8	A	$C_{34}H_{39}N.HCl$	76.25	76.84	10.67	10.43[a]
3	$(C_6H_{11}CH_2)_2CHNHCH_2C_6H_4$-*p*-$OCH_3$	150	53	7.8	A	$C_{33}H_{41}NO.HCl$	73.58	73.25	10.38	10.14
4	(1-$C_{10}H_7CH_2$)$_2CHNHCH_2C_6H_5$	238	75	..	A	$C_{38}H_{37}N.HCl$	82.26	82.16	6.44	6.53
5	$(C_6H_5CH_2)_2CH$-NHC_3H_6- 	230	64	..	B	$C_{28}H_{29}N_3O_2.HCl.{}^1/_2H_2O$	69.84	70.12	6.47	6.33[b]
6	$(C_6H_5)_2CHCH_2NHCH_2C_6H_5$[c]	260	20	7.6	A	$C_{21}H_{21}N.HCl$	77.87	77.62	7.01	6.86
7	$(C_6H_5)_2CHCH_2NHCH_2C_6H_4$-*p*-$OC_5H_{11}$	217	62	..	A	$C_{26}H_{31}NO.HCl$	76.17	76.23	7.87	8.00
8	$[(C_6H_5CH_2)_2CHNHCH_2C_6H_4O]_2$-$C_3H_6$	238	46	..	A	$C_{43}H_{52}N_2O_2.2HCl$	75.28	75.39	7.26	6.98

[a] Calcd.: N, 3.71. Found: N, 3.55. [b] Calcd.: N, 6.52. Found: N, 6.51. [c] The primary base was prepared by the reduction of diphenylacetonitrile by the method of Benoit, G., Delavigne, R., and Eliopoulo, P., *Ann. pharm. franç.*, **10**, 181 (1952).

Dry hydrogen bromide gas was passed into 75 Gm. (0.35 mole) of dibenzylcarbinol at 100–120° until the gas was no longer absorbed. The layers were separated. The bromide layer was washed with water and sodium bisulfite solution. After drying over anhydrous calcium chloride, the bromide was distilled; 60 Gm. (62%), b. p. 140–142° at 1 mm.

A solution of 19 Gm. (0.082 mole) of once distilled α-benzylphenethyl bromide and 9 Gm. (0.084 mole) of benzyl amine in 150 ml. of absolute ethanol was heated at reflux for fourteen hours. Upon cooling, 9 Gm. (32%) of white crystals, m. p. 268–269°, was obtained. This solid was converted to the hydrochloride and proved identical to I, 3.

Penicillin salts.—A solution of one equivalent of potassium penicillin G in a minimum of water was added, dropwise, with stirring to a solution of the hydrochloride salt of the α-benzylphenethylamine in a water-miscible solvent such as alcohol or N,N-dimethylformamide. The mixture was then cooled to separate the crystalline penicillin salt. The product was usually washed well with water and analyzed after thorough drying. Several were recrystallized from aqueous ethanol at room temperature.

α - Benzyl - N - (3 - phenylpropyl) - phenethylamine (Table I, 5).—Recrystallization of the hydrochloride mixture obtained from the reductive alkylation of α-benzylphenethylamine by cinnamaldehyde (*Method A*) gave considerable quantities of a white crystalline by-product, m. p. 245–246°. A satisfactory analytical sample could not be obtained by repeated crystallization from various alcohols.

Anal.—Calcd. for $C_{24}H_{25}N.HCl$: C, 79.21; H, 7.20. Found: C, 78.60; H, 7.59.

The hydrochloride was neutralized by shaking in ether-sodium hydroxide solution until clear. The ether extract was separated, dried, and concentrated. Distillation of the residue gave a colorless liquid with a distinctive amine odor, b. p. 172–173° at 0.5 mm.

Anal.—Calcd. for $C_{24}H_{25}N$: C, 88.03; H, 7.70; N, 4.28. Found: C, 88.18; H, 7.73; N, 4.31.

This liquid gave positive bromine and potassium permanganate tests for unsaturation and appeared to be N-(α-benzylphenethyl)-cinnamylamine.

Another preparation of I, 5 using hydrocinnamaldehyde as alkylating agent gave a 62% yield of saturated base, b. p. 200–201° at 0.8 mm. A forerun of unreacted aldehyde was also collected.

TABLE IV.—PENICILLIN SALTS

No.	Amine	M. P.	Yield	Solubility, %	Potency[a] u./mg. Calcd.	Found	Analysis[b] Carbon Calcd.	Found	Hydrogen Calcd.	Found	Nitrogen Calcd.	Found
1	I, 3	121	94	0.076	934	907	71.78	71.28	6.50	6.59	6.61	6.82
2	I, 5	119	91	0.020	875	862	70.46	70.89	6.95	6.95	..	..
3	I, 9	116	95	0.060	..	..	72.08	71.26	6.66	6.93	..	..
4	I, 10	138	95	0.022	..	..	72.64	72.59	6.79	6.88	..	..
5	I, 11	116	90	0.056	892	755	70.35	69.16	6.51	6.74	6.31	6.24
6	I, 12	130	95	0.047	..	..	70.66	71.12	6.67	6.34	6.18	6.34
7	I, 15	135	88	0.038	..	..	70.97	70.73	6.83	7.05	6.06	6.08
8	I, 16	143	96	0.033	..	..	70.97	70.93	6.83	6.99	6.06	5.93
9	I, 17	134	95	0.017	839	831	71.26	71.25	6.98	6.82	5.94	6.18
10	I, 18	143	90	0.026	..	..	71.26	71.86	6.98	7.39	5.94	5.49
11	I, 19	135	88	0.012	823	731	71.53	71.39	7.12	7.19	5.82	5.99
12	I, 21	135	85	0.019	..	..	71.53	71.38	7.12	7.27	5.82	5.70
13	I, 22	110	91	0.013	..	..	72.78	73.52	7.76	8.02	5.31	5.07
14	I, 23	141	95	0.009	800	758	72.75	72.35	6.51	6.51	5.66	5.51
15	I, 24	130	75	0.020	816	797	67.61	67.70	6.37	5.50	5.77	5.57
16	I, 25	128	80	0.066	..	..	68.09	68.21	6.01	6.18	6.27	6.10
17	III, 1	152	55	0.031	..	..	70.40	70.22	8.25	8.36	..	..

[a] Supplied by Dr. John Ehrlich and Mrs. Margaret Galbraith.
[b] The penicillin salts often were analyzed without recrystallization. Many were noted to contain water by Victor Meyer assay.

Anal.—Calcd. for $C_{24}H_{27}N$: C, 87.49; H, 8.26. Found: C, 87.40; H, 8.32.

Several other salts of I, 5 were prepared by shaking the base and appropriate acid in water for several hours. The salts were isolated, analyzed, and characterized; acetate (m. p. 102°), sulfamate (m. p. 159°), lactate (m. p. 70°), nitrate (m. p. 137°), and oxalate (m. p. 206°).

1-Phenethyl-3-phenylpropylamine (Table II, 2).—The Leuckart reaction on 1,5-diphenyl-3-pentanone (3) gave a 76% yield of colorless liquid, b. p. 162–173° at 1.4–1.8 mm. A sample was converted to the hydrochloride (2), m. p. 157–160°.

5-Phenyl-1-(4-phenylbutyl)-pentylamine (Table II, 7).—The Leuckart reaction on 1,9-diphenyl-5-nonanone[4] gave a 31% yield of yellow liquid, b. p. 177–178° at 0.7 mm. This slightly impure amine was used for further alkylations.

1,3-Di(α-naphthyl)-2-propanone.—A solution of 38.8 Gm. (0.97 mole) of sodium hydroxide pellets in 1,500 ml. of water along with 170.7 Gm. (0.97 mole) of crude α-naphthylacetic acid was prepared with stirring. After filtration, a solution of 71.3 Gm. (0.485 mole) of calcium chloride dihydrate in 500 ml. of water was added. The mixture was cooled and 155 Gm. (78%) of the calcium salt was separated by filtration.

The dried salt (155 Gm., 0.378 mole) was powdered and dry distilled to yield a liquid, b. p. 220–280° at 2–5 mm. which solidified upon cooling. The solid was recrystallized twice from ethanol to give 55 Gm. (47%) of fine needles, m. p. 110–111°.

Anal.—Calcd. for $C_{23}H_{20}O$: C, 89.00; H, 5.85. Found: C, 89.22; H, 6.04.

α-(α-Naphthylmethyl)-α-naphthylethylamine.—The Leuckart reaction on the ketone gave a 50% yield of colorless crystals, from 70% ethanol; m. p. 105–106°. Hydrolysis of the intermediate formyl derivative was accomplished by refluxing in 100 ml. of concentrated hydrochloric acid and 200 ml. of ethanol.

Anal.—Calcd. for $C_{23}H_{21}N$: C, 88.71; H, 6.80. Found: C, 88.74; H, 6.95.

[4] A 92% yield, b. p. 193–196° at 0.7–0.8 mm., from the catalytic reduction of 1,9-diphenyl-1,3,6,8-nonatetraen-5-one.

α-Cyclohexylmethylcyclohexylethylamine.—A suspension of 211 Gm. (1.0 mole) of α-benzylphenethylamine in 500 ml. of purified dioxane with 5 Gm. ruthenium dioxide (8) was hydrogenated at 130–140° at 1,200–1,400 p. s. i. for eight hours. The catalyst was removed by filtration and the solvent evaporated *in vacuo*. The dark residue was distilled to give 158 Gm. (72%) of colorless liquid, b. p. 125–126° at 1.4–1.5 mm. A sample was refractionated, b. p. 98° at 0.5 mm.

Anal.—Calcd. for $C_{15}H_{29}N$: C, 80.65; H, 13.08. Found: C, 81.21; H, 12.23.

The hydrochloride was prepared with hydrogen chloride gas in ether. The white solid was recrystallized from water, m. p. 172–173°.

Anal.—Calcd. for $C_{15}H_{29}NHCl$: C, 69.33; H, 11.64; N, 5.39; Cl, 13.65. Found: C, 69.08; H, 11.79; N, 5.32; Cl, 13.65.

N, 2-Dibenzyl-3-phenylpropylamine (Table III, 6).—A solution of 4.0 Gm. (0.018 mole) of α-benzylcinnamaldehyde (9) and 1.9 Gm. (0.018 mole) of benzylamine in 50 ml. of ethanol was heated at reflux for one hour. The solution was hydrogenated with 5% palladium-on-charcoal at room temperature and three atmospheres. After filtration and evaporation *in vacuo*, the residue was dissolved in ether and saturated with dry hydrogen chloride gas to give, after recrystallization from methanol, 2.2 Gm. (35%) of white crystals, m. p. 144–146°.

Anal.—Calcd. for $C_{23}H_{24}ClN$: C, 78.50; H, 7.45. Found: C, 78.11; H, 7.46.

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An Improved Method for the Determination of Lanthionine*

By M. X. SULLIVAN and CHESTER F. MIJAL†

SUBSEQUENT to the discovery of cystine by Wollaston (1) in 1810 in a urinary calculus, a great deal of work was done as to its nature and occurrence. Baudrimont and Malaguti (2) found that cystine contained sulfur, which previous workers had missed. Then Mörner (3) isolated cystine from the hydrochloric acid hydrolyzate of horn. Subsequently, cystine was found widespread in proteins by a host of investigators.

Dependent on the method of purification of the particular protein, the procedure of isolation of cystine, and its determination, great variation was found in the cystine content of individual proteins. Hoffman (4), in 1925, reported that hair treated with hot sodium carbonate (1 to 4 per cent) for a short time lost about 24 per cent of its sulfur. The treated hair was identical in appearance with that which had not been treated. However, although the treated hair retained about 75 per cent of its original sulfur, no cystine could be isolated from an acid hydrolyzate. About the same time Sullivan (5), employing his highly specific test for cystine, found that grain-curd casein, never in contact with alkali, had a higher cystine content than Hammarsten casein because the latter had gone through a sodium carbonate treatment. Later, Sullivan and Hess (6), using the Sullivan cystine test, found that treating various proteins with dilute alkali lowered the cystine content, and they (7) also found that treatment of insulin with pyridine lowered the cystine content of the hydrolyzed insulin. The pyridine treatment apparently did not affect the physiological unitage.

All our work pointed to the conclusion that treatment with weak alkali made changes in the nature of the protein, especially as to the amount of cystine found in an acid hydrolyzate.

In the meantime, Küster and Irion (8) reported the isolation of a thioether from the acid hydrolyzate of wool heated with sodium sulfide. Horn, Jones, and Ringel (9) were unable to repeat this isolation, but did succeed in isolating a thioether by boiling wool in a 2 per cent sodium carbonate solution, and hydrolyzing the treated wool with 20 per cent hydrochloric acid solution. On concentrating the hydrolyzate, dissolving the concentrate in absolute ethyl alcohol, and adding pyridine to the alcohol extract, they obtained a certain amount of a thioether which they named "lanthionine" and found to be structurally ($COOH\text{-}CHNH_2\text{-}CH_2S\text{-}CH_2\text{-}CHNH_2\text{-}COOH$)—a symmetrical thioether. The structure was confirmed by du Vigneaud and Brown (10) by synthesis of *meso*-lanthionine from *l*-cysteine and methyl *d,l*-α-amino-β-chloropropionate hydrochloride in strongly alkaline solution. Horn and Jones (11) then isolated lanthionine from human hair, chicken feathers, and lactalbumin, after treatment with sodium carbonate, and du Vigneaud and co-workers (12) found that amorphous insulin, similarly treated, yielded lanthionine. Others who have worked on the formation of lanthionine in a detailed, comprehensive way are Cuthbertson and Phillips (13) and Lindley and Phillips (14).

Lanthionine resembles methionine in that it is a thioether. Baernstein (15) had shown that hydriodic acid containing KH_2PO_2 (potassium hypophosphite) converts methionine into the thiolactone of homocysteine,

$$CH_2SCH_2CH_2CHNH_2COOH + HI = \begin{matrix} & S & \\ & \diagdown\!\diagup & \\ CH_2 & & CHNH_2C = O \\ | & & | \\ CH_2 & & \end{matrix}$$

Later, Hess and Sullivan (16) considered that one mole of lanthionine heated with hydriodic acid should yield 1 mole of cysteine, and developed a method for the quantitative determination of lanthionine. Lanthionine plus a 57 per cent solution of hydriodic acid, reinforced with potassium hypophosphite (KH_2PO_2) to prevent liberation of iodine, was hydrolyzed at 135–140° for four hours under a steady stream of nitrogen. A quantitative splitting resulted, with rupture of the carbon-sulfur bond of lanthionine and with the formation of cysteine.

The Sullivan (15) procedure for the estimation of cysteine was then applied. Lanthionine, as such, does not react in the Sullivan cysteine or cystine reaction; hence, if either of the latter compounds is present, it could be determined first, after hydrochloric acid hydrolysis, and then

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Taken in part from a thesis submitted by Chester F. Mijal in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Georgetown University, Washington, D. C.
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again, following hydrolysis with hydriodic acid, which yields only cysteine. The amount of lanthionine present would be measured by the increase in cysteine found after the hydriodic acid hydrolysis (*i.e.*, hydriodic acid hydrolysis minus hydrochloric acid hydrolysis). Multiplying the figure for the excess cysteine by 1.72 gives the amount of lanthionine.

The procedure of Hess and Sullivan for determining lanthionine is rather long, involves two separate hydrolyzates, and requires a special hydriodic acid. Accordingly, an investigation was made as to the possibility of splitting the lanthionine by cyanogen bromide, since the latter had been used by Cahours (17) to split such thioethers as dimethyl sulfide (CH_3SCH_3) by heating the thioether with solid cyanogen bromide in a sealed tube. Cahours considered that the reaction products were methyl thiocyanate (CH_3SCN) and a bromide of trimethyl sulfine [$(CH_3)_2SBr$]. Later, von Braun and Engelbertz (18) reported that the reaction is:

$$(CH_3)_2S + BrCN = CH_3SCN + CH_3Br$$

and that, as an intermediary product, [$(CH_3)_2$-$CNSBr$] may occur.

In the light of the work by Cahours and especially by von Braun and Engelbertz, Sullivan and Folk (19) studied the action of cyanogen bromide on lanthionine isolated from hair by the procedure of Horn, Jones, and Ringel (9). Sullivan and Folk found that lanthionine, heated with excess sodium cyanide and cyanogen bromide, would react in the Sullivan cysteine reaction as cysteine does. Lanthionine treated with sodium cyanide or cyanogen bromide (*separately*) does not give the Sullivan cysteine reaction. Also, when cysteine or cystine is similarly heated with sodium cyanide and cyanogen bromide, no evidence of cysteine could be obtained. The free amino acids are either destroyed by the procedure or are changed to some compound not reacting as cysteine does. The Sullivan and Folk (19) procedure was offered as a test for lanthionine.

However, the procedure of alternately heating the lanthionine-sodium cyanide-cyanogen bromide mixture in an open tube is somewhat tedious and requires care in manipulation. Accordingly, we sought an easier method. Such a method was devised and is herein given.

EXPERIMENTAL

Procedure —To 5 ml. of a 0.1 *N* hydrochloric acid solution containing 0.05–0.5 mg. of lanthionine in a 20 x 150 mm. test tube, add 1 ml. of 5% sodium cyanide solution. Shake gently and add 1 ml. of cyanogen bromide.[1] Stopper the test tube and heat for five minutes in a water bath held at 70°. Cool in cold water. To the cooled solution add 1 ml. of freshly prepared 0.5% aqueous solution of sodium 1,2-naphthoquinone-4-sulfonate. Shake the test tube for forty seconds and add 5 ml. of an alkaline sodium sulfite solution (10% of sodium sulfite in 0.5 *N* sodium hydroxide), stopper the tube tightly, and place it in the 70° water bath for fifteen minutes. Then cool in cold water and add 1 ml. of freshly prepared 2% sodium hydrosulfite ($Na_2S_2O_4$) in 0.5 *N* sodium hydroxide. Read the density of the color in the Klett-Summerson photoelectric colorimeter with the aid of the green filter (filter 54).

Preparation of a Standard Curve —The precision and reproducibility of the quantitative lanthionine procedure as outlined above were studied. Ten duplicate samples of lanthionine solution of each concentration shown in Table I were run. The standard deviation and percentage standard deviation were calculated. The standard curve is shown in Fig. 1.

The reddish color produced fades quite rapidly, as may be seen in Fig. 2. Accordingly, after the addition of the sodium hydrosulfite, the interval of elapsed time before reading the absorbance in the Klett-Summerson colorimeter should be the same for all tubes.

TABLE I.

mg.	Av. Reading	Sum Diff.[2]	S. D.	S. D. mg.
0.05	31	29	1.78	±0.002
0.1	60.3	50	2.36	±0.004
0.2	121.2	161	4.31	±0.006
0.4	240.6	264	5.41	±0.009

Determination of Lanthionine Produced in Wool —As a check on the method, a sample of white wool of unknown previous treatment (but possibly bleached) was employed. Thus 1 Gm. of the wool in question was boiled for thirty minutes with 200 ml. of a 2% solution of sodium carbonate. The treated wool was filtered on a sintered-glass funnel and washed with 200 ml. of distilled water. After being air-dried, the wool was hydrolyzed with 50 ml. of 20% hydrochloric acid solution until it no longer gave a biuret test. An equal volume of distilled water was added, and the solution (which was slightly colored) was treated with an iron-free Norit, boiled for two minutes, and filtered on a small Büchner funnel. The Norit was washed with 20 ml. of 0.1 *N* hydrochloric acid, and the washings were added to the main filtrate. The pH of the clear, colorless solution was adjusted to 3.5 with 5 *N* sodium hydroxide added dropwise with stirring, and the solution was transferred quantitatively to a 200-ml. volumetric flask and brought to volume by addition of 0.1 *N* hydrochloric acid.

The quantitative lanthionine determination was made by taking separately (*a*) a reagent blank (5 ml. of 0.1 *N* hydrochloric acid); (*b*) a hydrolyzate blank (1 ml. of the wool hydrolyzate plus 4 ml. of 0.1 *N* hydrochloric acid); (*c*) a standard control (2 ml. of standard lanthionine solution containing 0.2 mg. of lanthionine plus 3 ml. of 0.1 *N* hydrochloric acid);

[1] The cyanogen bromide is prepared by adding a 5% solution of sodium cyanide dropwise to a saturated, aqueous solution of bromine until a colorless solution results.

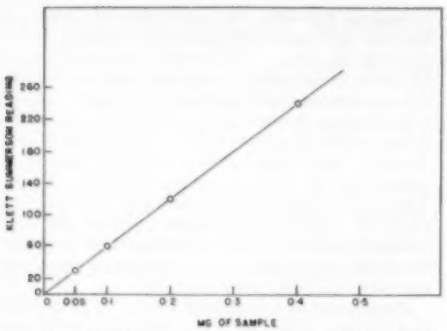


Fig. 1.—Standard curve, average of 10 duplicate samples.

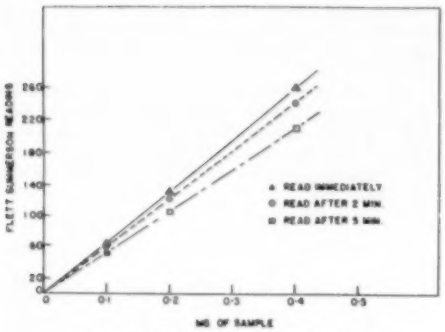


Fig. 2.—Fading of color with time.

and (*d*) the hydrolyzate (1 ml. of the wool hydrolyzate plus 4 ml. of 0.1 *N* hydrochloric acid).

To each of the four test tubes was added 1 ml. of 5% aqueous sodium cyanide solution, followed by 1 ml. of the cyanogen bromide solution. The tubes were stoppered, kept in the water bath at 70° for five minutes, and then cooled to 15° in cold running water. Successively, to each tube (with the exception of the hydrolyzate blank where water was substituted) was added 1 ml. of sodium 1,2-naphthoquinone-4-sulfonate solution. The test tube was stoppered and shaken for forty seconds. Then, 5 ml. of sodium sulfite solution was added to each tube and, after being restoppered, the tubes were placed in the water bath, kept at 70° for fifteen minutes, and cooled to 15°. In succession, one ml. of the sodium hydrosulfite ($Na_2S_2O_4$) solution was added and, after shaking, the absorbance was read immediately.

The following readings were obtained: (*a*) reagent blank 21, (*b*) hydrolyzate blank 10, (*c*) standard control 182, and (*d*) hydrolyzate 97.

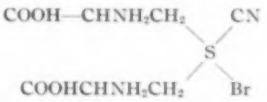
The calculations are: (*a*) standard control minus the reagent blank equals 161, and (*b*) the hydrolyzate minus the sum of the hydrolyzate blank and the reagent blank equals 66. These results indicate the presence of 0.082 mg. of lanthionine per ml. of the wool hydrolyzate, or 1.64% of lanthionine in this sample of alkali-treated wool.

DISCUSSION

The new procedure presented herein is a better colorimetric method for the quantitative determination of lanthionine, since it is performed more easily and obeys Beer's law over a wide range. It is somewhat different from that of Sullivan and Folk who, by strong heating, split the carbon-sulfur bond with the formation of a free sulfhydryl group (-SH), as shown by a positive nitroprusside test and by the cysteine estimated by the Sullivan cysteine reaction performed at room temperature. In the new reaction, no sulfhydryl (-SH) group is brought into play since the nitroprusside reaction is negative. Also, whereas Sullivan and Folk developed a color reaction at room temperature, the new procedure gives no color at room temperature on addition of the sodium cyanide, naphthoquinone derivative, and alkaline sodium sulfite solution. In short, the color only develops, with these reagents, on heating at 70°.

The specificity of the new lanthionine reaction is good. Related thioethers, such as thiodiglycolic acid ($COOHCH_2SCH_2COOH$), methionine (CH_3S-CH_2-CH_2CHNH_2COOH), and cystathionine ($COOH$-$CHNH_2CH_2CH_2SCH_2CHNH_2COOH$), treated with sodium cyanide and cyanogen bromide, gave no color. Free cysteine and free cystine are oxidized at once by cyanogen bromide and no longer give a nitroprusside reaction with or without sodium cyanide and became negative in the regular Sullivan reactions for cysteine or cystine.

Based on the work of von Braun and Engelbertz (18), it is possible that, in the lanthionine treatment, there is first formed a complex such as:



which split into $COOH$-$CHNH_2CH_2Br$ and $COOH$-$CHNH_2CH_2SCN$. The RSCN complex, on heating to 70° with sodium cyanide, the naphthoquinone derivative, sodium sulfite, and alkali, would be converted to a disulfide as suggested by the findings of Kaufmann (20) in the case of thiocyanates of fatty acids. However, the full explanation of the lanthionine reaction has not been ascertained.

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Amine Derivatives of Cyanuric Chloride IV*

Catalytic Hydrogenolysis of Aromatically-Bound Chlorine

By WILLIAM O. FOYE and MELVIN H. WEINSWIG†

A method has been found whereby aromatically-bound chlorine may be reduced from cyanuric chloride derivatives. This involves catalytic hydrogenolysis with 10 per cent palladium-on-charcoal at atmospheric pressure. A series of mono- and diamino-*s*-triazines was prepared by this procedure without rupture of the *s*-triazine or other aromatic rings. Two of the diamino-*s*-triazines caused decreases of blood glucose in rats but showed no diuretic activity in dogs.

CYANURIC CHLORIDE is a convenient intermediate for the preparation of mono-, di-, or tri-substituted *s*-triazines, since stepwise replacement of the three chlorine atoms is possible (1). If a method were available where the unreacted chlorine atoms could be removed from the monoaminodichloro or diaminomonochloro derivatives so obtained, a simple means of preparing monoamino-*s*-triazines and diamino-*s*-triazines, or formoguanamines, would be available. Lipschitz and Hadidian (2), Clauder and Bulcsu (3), and Shapiro, *et al.* (4), have shown formoguanamine and its derivatives to be powerful diuretics. Their compounds, however, were obtained by rather tedious cyclization procedures which were unsuitable for preparing a number of triazine derivatives including the aminopyridyl and aminothiazolyl. Modest (5) has also depended upon a cyclization procedure to obtain aminodihydro-*s*-triazines which showed antivitamin, antimalarial, and antitumor activities.

Various attempts have been made to remove the chlorine atoms from cyanuric chloride derivatives to achieve a more convenient synthesis of mono- and disubstituted-*s*-triazines. Hirt, Nidecker, and Berchtold (6) reported the reduction in very small yields of 2-alkylamino-4,6-dichloro-*s*-triazines to 2-alkylamino-*s*-triazines using a palladium-on-charcoal catalyst. This is the only instance in the literature of the successful catalytic reduction of an *s*-triazine. Grundmann, *et al.* (7), and Burger and Hornbaker (8) were unable to hydrogenolyze the chlorines of cyanuric chloride using nickel and noble metal catalysts. The latter found also that the reduction of cyanuric chloride with lithium aluminum hydride gave only 2-dimethylamino-4,6-dichloro-*s*-triazine, which they rationalized as occurring through hydrogenolysis of the ring to form dimethylamine which then reacts with unchanged cyanuric chloride. Chafetz (9) later supported this hypothesis by isolating dimethylamine from this reaction. Grundmann (10) has explained the failures of catalytic hydrogenation attempts by showing that *s*-triazine is a powerful noble metal catalyst poison.

The catalyst selected for the present attempt toward removal of ring-halogen from aminochloro-*s*-triazines by hydrogen was a commercially prepared 10 per cent palladium-on-charcoal catalyst previously found suitable for reduction of amino acid chlorides to amino aldehydes (11). It was successful in catalyzing the removal of chlorine from cyanuric chloride derivatives without rupture of the *s*-triazine ring, and the yields of amino-*s*-triazines obtained ranged from 28 to 82 per cent. The reactions were carried out at atmospheric pressure and temperatures of 60–80° by bubbling hydrogen through a suspension of the catalyst and either a solution or suspension of the chloro-*s*-triazine. The hydrogen chloride formed was bound by the amino-*s*-triazine, and excess hydrogen chloride could be titrated in the evolved gases. That other aromatically-bound halogen is removed by this procedure is shown by the loss of the chloro group from the *p*-chloroanilino substituents. The physical properties of the products obtained by this procedure are recorded in Table I.

Some difficulties with elemental analyses were encountered with these compounds as has been noted with other *s*-triazine derivatives (12). In these cases, which are probably due to depolymerization to cyanogen which reacts with the copper oxide in the combustion tube, analysis for ionizable chloride by the Volhard procedure generally gave results approximating the theoretical. Water determinations by loss of weight

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Abstracted from the thesis submitted by M. H. Weinswig as a requirement for the degree of Master of Science, 1957.
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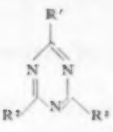
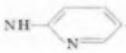
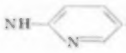
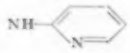
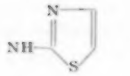
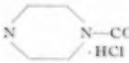
The authors are indebted to Drs. K. K. Chen, Mary A. Root, and E. B. Robbins of the Lilly Research Laboratories for carrying out the diuretic and blood sugar tests and providing the results for publication. Appreciation is also acknowledged for financial assistance from the White Laboratories, Inc.

TABLE I.—AMINO-*s*-TRIAZINES



R′	R²	R³	Yield, %	M.p.[a], °C.	Formula	Analyses, %[b] Calcd.	Found
$NH_2 \cdot HCl$	NH_2	H	44	>300	$C_3H_6N_6Cl$	C: 24.41	24.46
						H: 4.10	4.25
$NH—(CH_2)_2—NH—CO_2C_2H_5 \cdot HCl$	$NH—(CH_2)_2—NH—CO_2C_2H_5$	H	82	143–146	$C_{12}H_{24}N_7O_4Cl \cdot 2H_2O$	C: 38.25	37.81
						H: 6.93	6.87
						H_2O: 8.7	8.6[c]
$NH—C_6H_5 \cdot HCl$[e]	H	H	30	250–252	$C_9H_8N_4Cl$	Cl: 17.02	16.95[d]
$NH—C_6H_5 \cdot HCl$[e]	$NH—C_6H_5 \cdot HCl$	H	28	>300	$C_{15}H_{13}N_5Cl_2$	Cl: 21.13	20.84
$NH_2 \cdot HCl$[e]	$NH—C_6H_5 \cdot HCl$	H	74	170–173	$C_9H_{11}N_5Cl_2$	Cl: 27.54	27.11
	H	H	35	238–241	$C_8H_9N_4Cl_3 \cdot 4H_2O$	C: 30.92	30.19
						H: 5.35	5.35
						H_2O: 22.6	22.2
		H	32	219–220	$C_{13}H_{12}N_7Cl \cdot 4H_2O$	C: 41.71	40.09
						H: 5.71	5.62
						Cl: 9.52	9.47
						H_2O: 19.3	19.8
	H	H	47	>300	$C_5H_6N_4SCl$	Cl: 16.51	16.45
		H	33	>300	$C_5H_6N_7S_2Cl$	Cl: 11.18	10.99
	H	H	77	220–222	$C_{18}H_{16}N_4O_2Cl$	C: 43.92	43.91
						H: 5.87	6.22
		H	30	294–295	$C_{17}H_{23}N_7O_4Cl$	C: 48.71	48.95
						H: 6.54	6.76

[a] The melting points were taken on a Fisher-Johns block and are uncorrected.
[b] The carbon-hydrogen analyses were obtained from the Weiler and Strauss Microanalytical Laboratory, Oxford, England.
[c] Measurements of water content were done by determining loss of weight at 100° *in vacuo* (2–5 mm.) over phosphorus pentoxide.
[d] Determinations of ionizable chloride were done by the Volhard method.
[e] The *p*-chloroanilino groups in these compounds were reduced to anilino groups.

after vacuum drying were also carried out where indicated.

BIOLOGICAL RESULTS

Three of the diamino-*s*-triazines were tested at the Lilly Research Laboratories for diuretic and blood glucose effects. These compounds, 2,4-bis-(2′-carbethoxyaminoethylamino)-*s*-triazine hydrochloride, 2,4-bis-anilino-*s*-triazine dihydrochloride, and 2,4-bis-(4′-carbethoxypiperazino)-*s*-triazine hydrochloride showed no significant diuresis when tested orally in dogs at dose levels of 5–20 mg./Kg. The bis-anilino derivative caused an 18 mg. % decrease in blood glucose, lasting less than three hours, at a dose of 250 mg./Kg. in rats, p. o., and the bis-carbethoxypiperazinyl derivative caused a 14 mg. % decrease in blood glucose in seven hours at the same dose level in rats. The third *s*-triazine showed no effect on blood glucose at this level.

EXPERIMENTAL

Aminochloro-*s*-triazines.—2,4-Diamino-6-chloro-*s*-triazine was supplied by the American Cyanamid Co. 2-Chloro-4,6-bis-(2′-carbethoxyaminoethylamino)-*s*-triazine was prepared by Dr. Lester Chafetz (12d). 2,4-Dichloro-6-(2′-pyridylamino)-*s*-triazine, 2-chloro-4,6-bis-(2′-pyridylamino)-*s*-triazine, 2 4-dichloro-6-(2′-thiazolylamino)-*s*-triazine,

and 2-chloro-4,6-bis-(2′-thiazolylamino)-*s*-triazine were prepared according to Foye and Buckpitt (13). 2 - (4′ - Carbethoxypiperazinyl) - 4,6 - dichloro - *s* - triazine and 2,4-bis-(4′-carbethoxypiperazinyl)-6-chloro-*s*-triazine were prepared according to Foye and Chafetz (12d).

2,4 - Dichloro - 6 - *p* - chloroanilino - *s* - triazine was prepared according to Cuthbertson and Moffatt (14). 2-Chloro-4,6-bis-(*p*-chloroanilino)-*s*-triazine and 2 - amino - 4 - *p* - chloroanilino - 6 - chloro - *s* - triazine were prepared by the general procedure of Thurston (12a). The first of these two compounds was a white powder which melted at 243–245° and was obtained in 92% yield.

Anal.—Calcd. for $C_{15}H_{10}N_5Cl_3$: C, 49.21; H, 2.79. Found: C, 48.87; H, 2.93.

The latter compound, 2-amino-4-*p*-chloroanilino-6-chloro-*s*-triazine, was prepared by forming a slurry of cyanuric chloride (5.0 Gm., 0.027 mole) in 60 ml. of water and 60 ml. of dioxane at 0–5° and adding slowly a solution of *p*-chloroaniline (3.5 Gm., 0.027 mole) in 50 ml. of dioxane. An excess of aqueous sodium bicarbonate was added gradually, and the reaction mixture was stirred at 0–5° for two hours. To this slurry was then added 4.3 ml. (0.027 mole) of ammonia water, and the temperature was raised to 40–50°. The reaction mixture was stirred at this temperature for two hours, and the precipitate was filtered, washed free of chloride ion with ice water, and pressed as dry as possible. The product was dried overnight at 60°. After being washed with hot alcohol and redried overnight at 60°, 5.4 Gm. (78%) of a fine, white powder was obtained which decomposed at 200°.

Anal.—Calcd. for $C_9H_7N_4Cl_3$: C, 42.34; H, 2.77. Found: C, 41.93; H, 2.90.

Amino-*s*-triazines—The following is representative of the reduction procedure. 2,4-Diamino-6-chloro-*s*-triazine (6.0 Gm., 0.04 mole) was dissolved in 200 ml. of propylene glycol (95% ethanol was suitable for the other compounds reduced) with heating. The solution was placed in a three-necked flask fitted with a mechanical stirrer, an inlet tube for hydrogen, and an outlet tube for the hydrogen and other gases not absorbed by the amino-*s*-triazines. Six grams of 10% palladium-on-charcoal (American Platinum Works, Newark, N. J.), dried at 60°, was added to the solution and the temperature of the mixture was maintained at 70–78° during the reaction. Hydrogen was then passed into the stirred mixture for four hours. The evolved gases were bubbled through 0.2 *N* sodium hydroxide solution containing phenolphthalein to detect any hydrogen chloride or hydrogen cyanide liberated. The mixture, while still warm, was filtered to remove the catalyst.

The resulting solution was distilled under reduced pressure to give a residue of 2.7 Gm. (44%) of white 2,4-diamino-*s*-triazine hydrochloride which did not melt below 300°. The product, unlike the starting material, was soluble in alcohol, slightly soluble in water, and gave a positive test for ionizable halogen. Recrystallization of the products was generally successful from alcohols or petroleum ether-alcohol solutions.

SUMMARY

1. Mono- and diamino-*s*-triazines have been prepared in good yield by catalytic hydrogenolysis of halogen in the corresponding cyanuric chloride derivatives using 10 per cent palladium-on-charcoal. The method appears to be general, without causing rupture of the *s*-triazine or other heterocyclic rings, but suffers from the disadvantage that other aromatically-bound halogen is also removed.

2. The following new *s*-triazines were prepared and characterized: 2-chloro-4,6-bis-(*p*-chloroanilino), 2-amino-4-*p*-chloroanilino-6-chloro, 2,4-diamino hydrochloride, 2,4-bis-(2′-carbethoxyaminoethylamino) hydrochloride, 2-anilino hydrochloride, 2,4-dianilino dihydrochloride, 2-amino-4-anilino dihydrochloride, 2-(2′-pyridylamino) dihydrochloride, 2,4-bis-(2′-pyridylamino) hydrochloride, 2-(2′-thiazolylamino) hydrochloride, 2,4-bis-(2′-thiazolylamino) hydrochloride, 2-(4′-carbethoxypiperazinyl) hydrochloride, and 2,4-bis-(4′-carbethoxypiperazinyl) hydrochloride.

3. Three of the diamino-*s*-triazines were tested for oral diuretic activity and showed no significant effects. The bis-anilino and bis-carbethoxypiperazinyl derivatives caused decreases in blood glucose in rats, however.

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Determination of Rotenone in Pharmaceuticals*

By JACK T. BRYAN and WARREN R. LINCOLN

A procedure in which chloroform was used to extract rotenone from a pharmaceutical mixture has been described. A colorimetric assay for rotenone was adapted for use as an analytical tool in product development. Both moisture and heat of reaction were shown to be detrimental in the development of the color for assay.

THE DISCOVERY by Gross and Smith (1) that rotenone will react with sodium nitrite to give a stable red color under certain conditions has served as a basis for the development of a colorimetric assay. The method consisted of treating an acetone solution of rotenone with alcoholic potassium hydroxide and then acidifying with dilute nitric acid containing sodium nitrite. Goodhue (2) reported that this procedure was not entirely satisfactory because of an intense yellow background caused by the reagents. The condition was presumably obviated by Goodhue (2) by substituting sulfuric acid for nitric acid, by diluting the potassium hydroxide with water, and adding sodium nitrite with the alcoholic potassium hydroxide. In 1945, Jones (3) made further modifications to this later procedure in an attempt to obtain increased precision. It was recorded by Jones (3) that purity of the alcohol used to prepare alcoholic potassium hydroxide was important. Alcohol which had been distilled after refluxing with zinc and potassium hydroxide did not give background color. The temperature at which the red color was developed was shown to be of prime importance as well as the manner in which the acid was added to the alkaline solution of rotenone.

The colorimetric procedure for rotenone is not specific since the color can be developed with deguelin, dihydrorotenone, and other related compounds. The analytical methods reported above relate primarily to the assay of rotenone in agriculturally used insecticides. Our immediate concern was the need to adapt this method to the analysis of rotenone, in pure form, in pharmaceutical preparations containing several other active ingredients and vehicle components of the usual emulsion type. These pharmaceuticals are intended to be used in veterinary medicine for the treatment of certain dermatoses. Several modifications were made in the colorimetric determination reported by Jones (3) making it useful for such pharmaceutical products.

* Received March 27, 1959, from the Veterinary Research Laboratories of The Upjohn Co., Kalamazoo, Mich.

EXPERIMENTAL

Materials.—(*a*) Sodium nitrite solution: One gram sodium nitrite was dissolved in 10 cc. of water and diluted to one liter with 95% ethyl alcohol. (*b*) Potassium hydroxide, 40% solution: forty grams potassium hydroxide was dissolved in approximately 80 cc. of water, and after cooling, diluted to 100 cc. with water. (*c*) Alkaline sodium nitrite solution: one volume of 40% potassium hydroxide solution was mixed with 7 volumes of sodium nitrite solution. (*d*) Sulfuric acid, 9 *N*: free from nitrous acid. (*e*) Acetone, U. S. P. (*f*) Chloroform, U. S. P.

Apparatus.—Beckman, Model B, spectrophotometer with 1-cm. cells, water bath adjustable to 28° ± 0.5°.

Procedure.—An accurately measured sample containing approximately 1 mg. of rotenone was transferred to a 60-cc. separatory funnel. Distilled water was added to make a total volume of 5 cc. To this aqueous mixture was added 10 cc. of chloroform and the extraction carried out by shaking for several minutes. After the two phases separated, the chloroformic phase was separated and filtered through a pledget of glass wool into a 100-cc. volumetric flask. The extraction was carried out three times, after which the combined extracts were diluted to 100 cc. Several grams of dried sodium sulfate were added and the flask shaken, then allowed to stand for one hour.

A 10-cc. aliquot of the chloroformic extract was placed in a 25-cc. glass-stoppered Erlenmeyer flask. The chloroform was evaporated on a steam bath after which the flask was placed in a vacuum desiccator containing calcium chloride for a minimum of one hour. After drying, 2 cc. of acetone was added to the residue and the flask swirled gently until complete solution was effected.

A standard was prepared containing 100 μg. of rotenone in 2 cc. of acetone. A blank containing only 2 cc. of acetone was prepared and these were carried through the remaining procedure with the samples.

The flasks containing the sample, standard, and blank were placed in a constant temperature bath at 28° ± 0.5°. At the end of five minutes, 2 cc. of of alkaline sodium nitrite reagent was added and the flasks swirled to insure proper mixing. After reacting for five minutes, the flasks were removed to an ice bath, chilled for approximately one minute, and 5 cc. 9 *N* sulfuric acid was added rapidly from a volumetric pipet. Each flask was swirled vigorously until any solid which precipitated after the addition of the acid was dissolved. The flasks were then placed in the constant temperature water bath at 28° ± 0.5° for fifteen minutes. After the fifteen-minute interval, the color was read in a spectrophotometer at 545 mμ, using the blank to set the instrument. Samples which were cloudy were filtered through No. 5 Whatman filter paper before reading the color.

Calculations.—The mg./ml. of rotenone in the sample was calculated according to the following

formula: mg./ml. rotenone = $(Ax/Ast) \times (St/d)$ where Ax = observed absorbence of sample, Ast = observed absorbence of the standard, St = mg. of rotenone in standard, and d = dilution of sample.

Results.—The establishment of an optimum working range for the sample size was carried out by observing that samples of rotenone from 50 μg. to 200 μg. follow Beer's law. A sample size of 100 μg. was found to give a desirable color intensity. The stability of developed color with respect to time can be seen in Table I. The red color appears to be stable for a period of thirty to sixty minutes at room temperature after development.

TABLE I.—STABILITY OF COLOR, A_{545}

Time, min.	S_1	S_2	S_3
10	.342	.350	.350
15	.342	.352	.352
30	.339	.350	.350
60	.334	.348	.347
120	.327	.338	.338

The graph in Fig. 1 shows that the length of time for the development of color by the alkaline sodium nitrite is critical and the range of five minutes recorded in the literature (3) is within the range found in our laboratory.

Jones (3) has recorded that lowering the temperature of the alkaline nitrite mixture just prior to the addition of sulfuric acid increases the sensitivity of the color reaction. However, no investigation of the degree of sensitivity increase was recorded using an ice bath. Table II shows that the color intensity of the reaction is significantly increased by chilling for one minute just prior to the addition of sulfuric acid. After a cooling period of ten minutes, the color intensity decreases.

TABLE II.—EFFECT OF CHILLING NITRITE MIXTURE PRIOR TO ADDITION OF SULFURIC ACID, A_{545}

Time, min.	S_1	S_2	S_3
0 (no chilling)	.396	.400	...
1 (chilling)	.470	.472	.474
10 (chilling)	.459	.459	.461

In an attempt to further eliminate interfering variables, the effect of moisture in the final sample on the color intensity was studied. It was found that a greater degree of precision could be obtained between samples if the chloroformic extract containing the rotenone was dried over sodium sulfate before the aliquot for evaporation was removed. After the evaporation of chloroform, the flask containing the residue was placed in a vacuum desiccator for one hour. Table III shows the results.

TABLE III.—EFFECT OF MOISTURE IN SAMPLES, A_{545}

No Drying	Variation from Average, %	Dried	Variation from Average, %
.339	+14.1	.309	<0.1
.264	−11.1	.308	<0.1
.270	−9.3	.311	+0.2
.283	−4.7	.309	<0.1
.330	+11.1	.310	<0.1

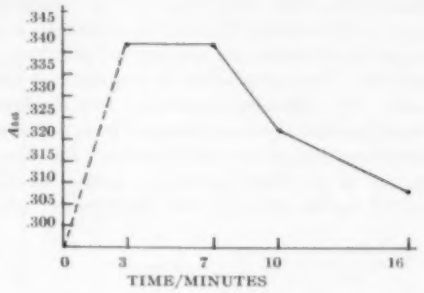

Figure 1.

Table IV further substantiates our findings that moisture in the final assay mixture causes a loss of precision and accuracy. These samples were assayed, either dry or with moisture present, then increments of rotenone were added and the samples reassayed for total amount of rotenone.

TABLE IV.—THE EFFECTS OF MOISTURE ON THE DETERMINATION OF ROTENONE

Sample	Found, mg./ml.	Added, mg./ml.	Total Found, mg.	Recovered, %
A moist	.625	0.50	1.214	117.7
A dried	.812	0.50	1.328	103.2
B dried	.661	1.00	1.655	99.4
C moist	.284	0.50	0.571	57.2
C dried	.262	0.50	0.773	101.8
D moist	.473	0.50	0.925	90.4
D dried	.519	0.50	1.013	98.8

Table V shows the results and standard deviation of a number of extractions and rotenone determinations using the method previously outlined. This table also shows that it was quite possible to quantitatively extract rotenone from a typical lotion-type pharmaceutical.

TABLE V.—DETERMINATION OF ROTENONE IN A PHARMACEUTICAL MIXTURE

Sample	Theory, mg./ml.	Found, mg./ml.	Theory, %
1	1.04	1.04	100
2	1.04	1.02	98
3	1.04	1.00	96
4	1.04	1.05	101
5	1.04	1.03	99
6	1.04	1.06	102
7	1.04	1.02	98
8	1.04	1.03	99
9	1.04	1.03	99
10	1.04	1.02	98
11	1.04	1.02	98
12	1.04	1.01	97
13	1.04	1.02	98
Standard deviation	...	1.03 ± 0.02	99 ± 2

SUMMARY AND CONCLUSIONS

An extraction procedure in which chloroform removed rotenone and other materials from an emulsion-type lotion has been described. The

chloroform-soluble ingredients of the emulsion, polyoxyl 40 stearate, glyceryl monostearate, and active constituents, did not appear to interfere with the subsequent color development of the assay. The following conclusions were reached: It was possible to extract rotenone from a typical pharmaceutical mixture of ingredients for external use in veterinary medicine; drying the extracted sample prior to color development produced a more stable and reproducible color reaction; and the use of an ice bath for a short chilling period to dissipate the heat of reaction caused by the addition of sulfuric acid increased the sensitivity of the color reaction.

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The Effect of Mescaline and Yohimbine on the Respiration of Rat Brain Homogenate*

By PAUL SHATZKO and S. K. SIM

By manometric method using conventional Warburg apparatus, the oxygen uptake of rat brain homogenate in glucose, glutamate, and succinate was found to be inhibited by mescaline and yohimbine. Yohimbine inhibited the brain homogenate's respiration in glucose considerably more than it did the respiration in glutamate and in succinate. Mescaline inhibited the homogenate's oxygen uptake in glucose, in glutamate, and in succinate to a similar degree, when the drug and the substrate were added to the brain homogenate at the same time. This is in contrast to the lack of mescaline inhibition of the succinate respiration of chopped brain preparation which was incubated with the drug for two and one-half hours before addition of substrate, as reported by previous investigators.

THE *in vivo* effects in experimental animals and in man of psychotomimetic agents including mescaline and yohimbine have been reported and reviewed in recent years (1, 2, 3). Various investigators have also reported the effect of such agents on the respiratory activity *in vitro* of brain preparations of different animals (3, 4). Quastel and Wheatley (4) found that mescaline and certain other amines inhibited the oxygen uptake of rat brain (chopped whole brain) in glucose, pyruvate, glutamate, and in lactate, but not in sodium succinate. This was later confirmed by Schueler (5) using the same method, that is, by incubating the chopped brain preparation with the drug for two and one-half hours before adding the respiratory substrate in question. Schueler (5) and Stevenson and Sanchez (6) showed the antidotal effect of sodium succinate on mescaline psychosis in humans. Schueler (5) pointed out the rather quick onset of the *in vivo* effect of mescaline in the humans and that it was difficult to correlate it with the *in vitro* results they obtained on the brain preparation.

In the experiments to be reported here, we investigated the effect of mescaline and yohimbine on the oxygen uptake of rat brain homogenate, using glucose, glutamate, and succinate as respiratory substrates, when the substrate and the drug were added to the brain preparation at the same time.

MATERIALS AND METHODS

Wistar albino rats weighing 180–230 Gm. were decapitated, the brain quickly removed, weighed, rinsed in buffer, and homogenized with a motor-operated Teflon pestle in a glass tissue grinder in Krebs-Ringer phosphate buffer, pH 7.3 (7). The tissue grinder and the buffer solution were chilled immediately before use. The final homogenate was made up with the buffer to 10% w/v with respect to the fresh weight of the brain tissue. Oxygen uptake was measured by conventional Warburg apparatus at 38°. Each Warburg reaction vessel contained 1 ml. of the brain homogenate, a substrate, with or without the drug, all dissolved in the same buffer and with a total volume of 3 ml. The center well contained 0.2 ml. of 20% KOH absorbed on filter paper strip. The gas phase was air. The substrate and the drug were tipped in from separate side arms of the reaction vessel at zero time after fifteen-minute equilibration in the constant temperature bath. In each experiment 5–8 vessels contained the homogenate, substrate, and the drug, and a similar number of vessels contained homogenate and substrate without the drug. Manometric readings were taken at first at five-minute and sub-

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This investigation was supported in part by a grant from the Canadian Foundation for the Advancement of Pharmacy.
Part of this work was presented at the 5th Canadian Conference on Pharmaceutical Research, Edmonton, Alta., August 1958.
The nitrogen determinations were made with the technical assistance of C. Mezei and I. C. Caldwell.

sequently at ten-minute intervals up to ninety minutes.

The substrates used were glucose (0.033 *M*), sodium glutamate (0.2 *M*), and potassium and sodium succinate (0.03 *M*). The drugs used were mescaline hydrochloride (0.03 *M*, 0.09 *M*, and 0.18 *M*), and yohimbine hydrochloride (0.005 *M* and 0.0073 *M*). All concentrations of substrates and drugs refer to the final concentration in the reaction mixture.

Nitrogen content of the homogenate was determined as total nitrogen by a micro-Kjeldahl method modified from those of Ballentine and Gregg (8) and of Pepkowitz and Shive (9).

The rate of oxygen uptake, Q_{O_2}, was based on the rate of the first forty minutes after zero time and calculated as μl. of oxygen per hour per mg. N. In a few cases, as indicated in the results, the rate of oxygen uptake was calculated as μl. of oxygen per hour per ml. of homogenate.

RESULTS

The oxygen uptake figures, at different time intervals, by 1 ml. of the brain homogenate in the presence of the substrate with and without the drug are plotted in Figs. 1, 2, and 3. Each oxygen uptake figure, in μl., is the mean of figures obtained from 5–18 reaction vessels in several typical experiments. Table I lists the rates of oxygen uptake as μl. of oxygen per hour per mg. of nitrogen except for those otherwise indicated as being μl. of oxygen per hour per ml. of homogenate. Although the brain homogenates used in different experiments on different days had different nitrogen contents ranging from 1.4 to 2.3 mg. N per ml. of homogenate, comparisons of the rates of oxygen uptake in the presence of any particular substrate with the drug and without the drug were made on the same batch of homogenate used on the same day. Therefore, the percentage of inhibition of rate of oxygen uptake by the drug would be the same whether this is calculated by

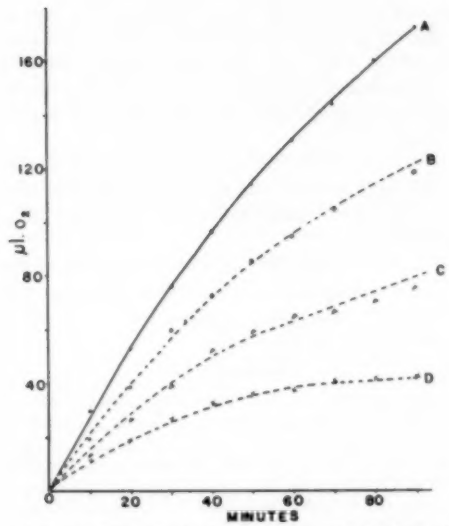

Fig. 1.—The effect of different concentrations of mescaline on oxygen uptake of rat brain homogenate in glutamate. A—glutamate (0.2 *M*); B, C, D—glutamate 0.2 *M* with mescaline 0.03 *M*, 0.09 *M*, and 0.18 *M*, respectively.

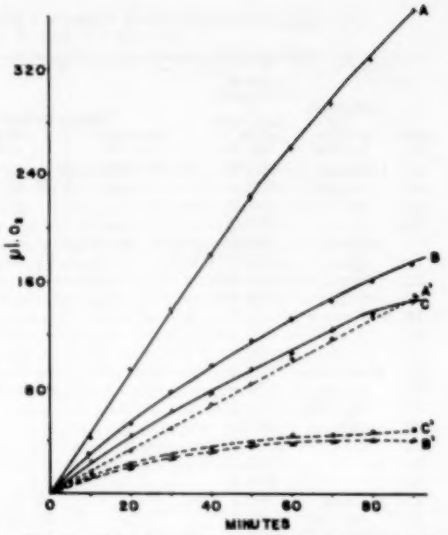

Fig. 2.—The effect of mescaline on oxygen uptake of rat brain homogenate in succinate, glutamate, and glucose. A—potassium succinate (0.03 *M*); A′—succinate + mescaline (0.18 *M*); B—glutamate (0.2 *M*); B′—glutamate + mescaline (0.18 *M*); C—glucose (0.033 *M*); C′—glucose + mescaline (0.09 *M*).

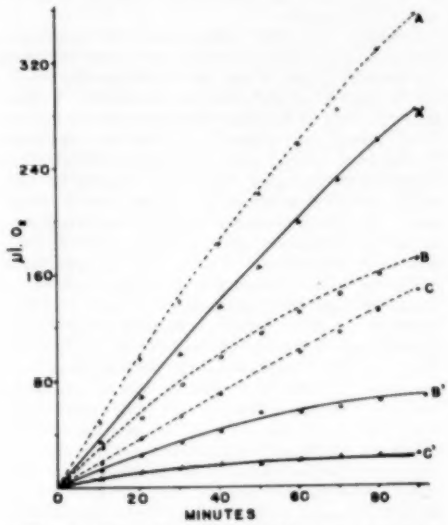

Fig. 3.—The effect of yohimbine on oxygen uptake of rat brain homogenate in various substrates. A—succinate (0.03 *M*); A′—succinate + yohimbine (0.0073 *M*); B—glutamate (0.2 *M*); B′—glutamate, + yohimbine (0.0073 *M*); C—glucose (0.033 *M*); C′—glucose + yohimbine (0.0073 *M*).

TABLE I.—THE EFFECT OF MESCALINE AND YOHIMBINE ON THE OXYGEN UPTAKE OF RAT BRAIN HOMOGENATE

Expt. No.	Substrate and Concentration	Q_{O_2} in Presence of Substrate μl./hr./mg. N	Q_{O_2} in Presence of Substrate plus Drug, % Inhibition in Brackets				
			Mescaline 0.03 *M*	Mescaline 0.09 *M*	Mescaline 0.18 *M*	Yohimbine 0.005 *M*	Yohimbine 0.0073 *M*
12	Glucose	107.1[a]	67.0[a] (37.4%)	.....	.....	.....	.....
28	0.033	103.1[a]	.....	37.1[a] (64.1%)	.....	.....	.....
26	*M*	40.2	.....	.....	.....	.....	6.7 (83.2%)
32		62.0	.....	.....	.....	.....	11.0 (82.3%)
13	Sodium	132.2[a]	103.0[a] (21.9%)	.....	.....	.....	.....
14	gluta-	134.9[a]	.....	77.9[a] (42.2%)	.....	.....	.....
22	mate	65.1	.....	.....	22.6 (65.2%)	.....	.....
39	0.2 *M*	89.8[a]	.....	.....	37.7[a] (58%)	.....	.....
16		53.7	.....	.....	.....	36.5 (32%)	.....
18		75.1	.....	.....	.....	.....	39.5 (47.4%)
19		76.8	.....	.....	.....	.....	37.8 (50.8%)
20	Potassium	139.0	.....	79.8 (42.5%)	.....	.....	.....
21	succin-	128.0	.....	81.8 (36.1%)	.....	.....	.....
24	ate 0.03	120.9	.....	.....	49.5 (59%)	.....	.....
23	*M*	129.6	.....	.....	.....	113.2 (12.6%)	.....
25		115.8	.....	.....	.....	.....	92.2 (20.3%)
27		130.2	.....	.....	.....	.....	103.6 (20.4%)
36		211.2	.....	.....	.....	.....	169.9 (19.6%)
37	Sodium	87.4	.....	48.7 (44.3%)	.....	.....	.....
38	succin-	82.6	.....	44.9 (46.1%)	.....	.....	.....
	ate 0.03 *M*						

[a] Calculated as μl./hr./ml. of homogenate.

comparing the rates as μl. of O_2 per hour per mg. N or by comparing the rates as μl. of O_2 per hour per ml. of homogenate.

It will be seen from Table I that both yohimbine and mescaline, at the concentrations used, inhibited the oxygen uptake of the brain homogenate respiring in glucose or glutamate or succinate. Yohimbine at 0.0073 *M* inhibited the respiration in glucose (82%) to a greater degree than it did the respiration in glutamate (50%) and in succinate (20%). The inhibition by mescaline of the respiration in the three substrates was less varied than in the case of yohimbine. At 0.09 *M* mescaline inhibited the respiration in glucose, glutamate, and succinate by 64%, 42%, and 42%, respectively. There was no significant difference in the inhibition of oxygen uptake by mescaline when using potassium succinate or sodium succinate as the substrate. As mentioned earlier, Quastel and Wheatley (4) and Schueler (5) found that mescaline inhibited the oxygen uptake of their rat brain preparation in glucose and in glutamate but not in sodium succinate. In their experiments, the brain preparation was of chopped whole brain and it was incubated with the drug for two and one-half hours before addition of the respiratory substrate. Our present results, with the use of brain homogenate, and with addition of mescaline and the substrate at the same time, show that mescaline inhibits the oxygen uptake in succinate as well as in glucose and in glutamate under these conditions. While one cannot readily correlate such *in vitro* experimental data with sodium succinate's antidotal effect on mescaline psychosis in humans, further studies on the effects of mescaline and other psychotomimetic agents on the respiratory activity of brain tissues would seem to be warranted as attempts toward elucidation of the mechanism of action of these agents.

SUMMARY

1. A study of the effects of mescaline and yohimbine on the oxygen uptake of rat brain homogenate with glucose, glutamate, and succinate as respiratory substrates has been made.

2. Mescaline at 0.09 *M* concentration was found to inhibit the brain homogenate's respiration in glucose, in glutamate, and in succinate by 64%, 42%, and 42%, respectively. At 0.18 *M*, mescaline inhibited the respiration in glutamate and in succinate by 65% and 59%, respectively.

3. The brain homogenate's respiration in glucose, in glutamate, and in succinate was inhibited by 0.0073 *M* yohimbine by 82%, 47%, and 20%, respectively.

4. The extent of mescaline's inhibition of the brain homogenate's respiration in succinate was essentially the same, whether potassium or sodium succinate was used.

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Diuretic Activity of Various Compounds as Determined by Urinary Excretion Studies in Rats*

By AMOS E. LIGHT

In the procedure described for evaluating diuretic activity in rats preliminary forced hydration of the animals may be omitted before the administration of the test dose which in itself does produce some hydration. The rats were starved overnight and the dose given orally or intraperitoneally in 0.5 ml. of water for each 100 Gm. weight. Three animals, each weighing 150–250 Gm., were dosed and grouped together in a metabolism cage resting on a polyethylene funnel for urine collection at four-hour and twenty-hour intervals. Values for urine volume, pH, sodium, and chloride were recorded. Ranges for the usual responses following water administration alone have been defined statistically for control values and amounts above these indicated typical diuretic responses. These actions were divided into two categories: natriuretic activity which increased sodium excretion in a large volume with high pH; and saluretic activity which may be further differentiated into: (*a*) pyrimidine, purine, and chlorothiazide types—immediate volume increase with saluresis; (*b*) mercurials—variable increased volume and sodium but decreased chloride excretion in twenty-hour period; and (*c*) miscellaneous types—saluretic activity in limited dose ranges such as with some triazines. Dose-response curves were obtained with known diuretics, and combinations were studied.

A DIURETIC has been defined as an agent that stimulates the secretion of urine; but since urine is composed of water, inorganic salts, and various organic compounds, all in changing amounts, this description appears to be rather ambiguous, especially when present-day interpretations are considered (1). Several different methods for evaluating diuretics in rats have been proposed (2–6). Many investigators have attempted to simulate the edema found in human pathology by forcing extra fluid into the animals, but this added treatment has been questioned (5). In the present study, certain specific excretory effects of accepted diuretics have been compared in both forcibly hydrated and in untreated animals. These included urine volume, pH, sodium, and chloride values with relation to the time of administration of the drugs. Other diuretics, individually and in combinations, have also been investigated in nonhydrated animals. Several new chemical derivatives were likewise tested for diuretic activity. Sex hormones and sulfhydryl binding compounds were also observed for effects on diuresis.

METHODS

In order to determine the types of diuretic responses suggested above, each dose of a compound under investigation was tested in groups of three male rats, each animal weighing 150–250 Gm. A single group was kept in a wire cage, 9 inches in diameter, placed over an 11-inch polyethylene funnel. A $^3/_{16}$-inch mesh stainless steel wire screen under the cage prevented fecal material from entering the beaker used to collect the urine sample. Water intake of the animals was determined by marking the water bottles at the conclusion of the various time periods. Spillage of drinking water into the urine sample was prevented by the introduction of a stainless steel trough under the regular water pan. This conducted any overflow away from the funnel opening and precluded any question as to the source of the urine volume especially when the compounds under test stimulated increased consumption of water.

Food was removed from the animals some twenty hours before the test, but water was always available unless noted. The drugs were usually given as solutions or suspensions in 0.5 ml. of water per 100 Gm. rat. Extra hydration was accomplished for certain comparative tests by the oral administration of 2.5 ml. water per 100 Gm. weight one hour before dosing and then 5.0 ml. per 100 Gm. weight at the time of dosing. During the remainder of the test period these forcibly hydrated animals did not have water available in the cage.

The urine volumes were measured for the periods indicated and the pH values determined in a pH meter[1] before appropriate dilutions, necessary for sodium and chloride quantitation. Sodium was determined in a flame photometer,[2] and chloride by the Volhard-Harvey technique (7).

In the diuretic screening tests of new compounds the fiducial limits of values obtained from control groups given water only, 0.5 ml. per 100 Gm. rat, were calculated; and the doses that produced values greater than these amounts were considered as having certain diuretic activities. These were compared with results from the known diuretics. Usually the excretory values of the four-hour and the following twenty-hour periods were obtained.

RESULTS AND DISCUSSION

As may be seen in Fig. 1, forced hydration along with acetazolamide[3] given intraperitoneally pro-

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[1] pH Meter, Model G., Beckman Instrument Co., South Pasadena, Calif.
[2] Flame Photometer, Process and Instruments, 15 Stone Ave., Brooklyn, N. Y.
[3] Diamox—Lederle Laboratories Div., American Cyanamid Co., Pearl River, N. Y.

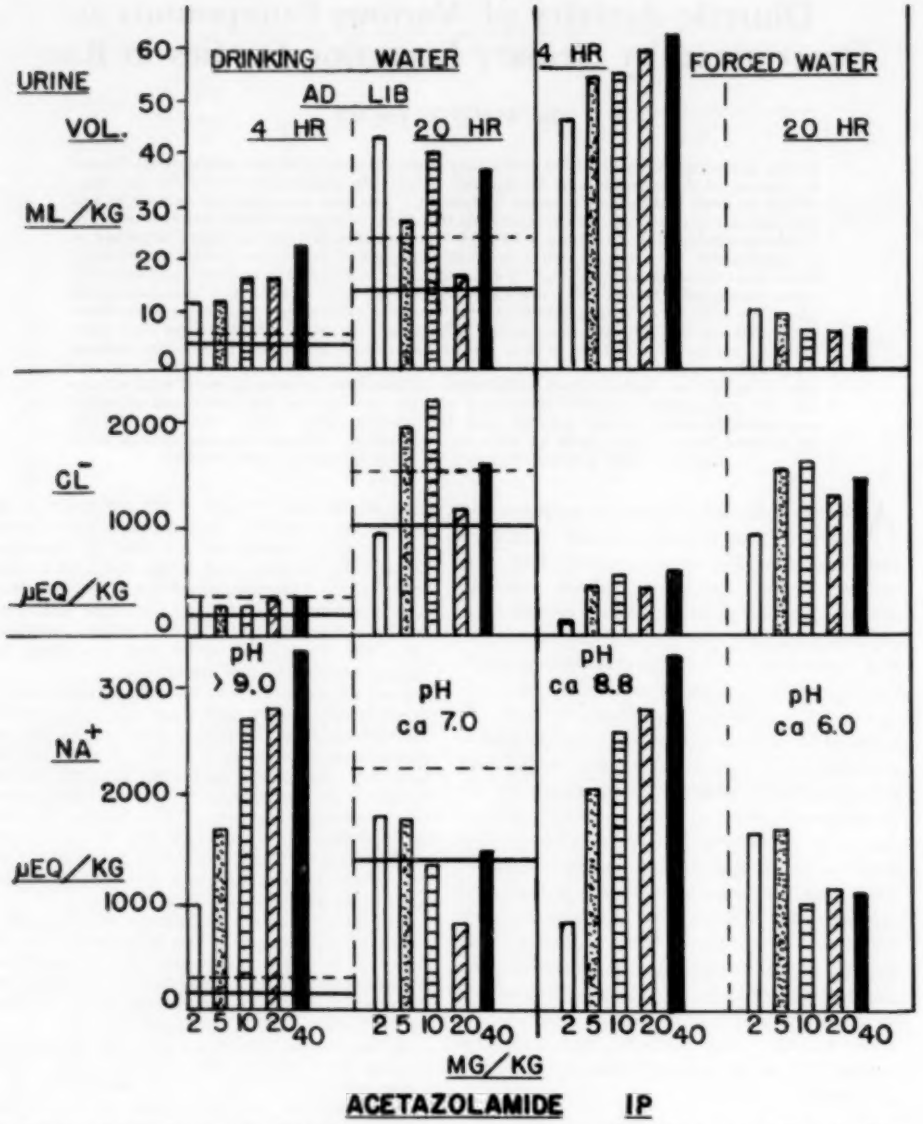

Fig. 1.—Urinary outputs produced by acetazolamide given intraperitoneally to rats with and without preliminary hydration. Heavy horizontal lines in first two sections represent average control values, and the broken lines above each are the fiducial limits (2 × S. D.). The pH values are recorded above the sodium bar graphs.

duced more urine volume at all dose levels than with the water given *ad libitum* during the first four hours. However, the actual increases in volumes from the lower doses to the higher ones were about the same in both procedures. In fact, the per cent increases were obviously much greater in the minimally treated animals. No significant difference was found between the procedures in comparable sodium, chloride, and pH values during the four-hour period. In both, pronounced natriuresis was exhibited with values of over 3,000 microequivalents of sodium per Kg. for this period. With the higher doses the pH likewise increased to 9 from a control range of 6–7. The chloride excreted, however, did not rise above the values usually found in the control group. These control values are summarized in Table I for convenience. The volume output for the forcibly hydrated animals was low during the twenty-hour period for these rats received no drinking water. On the other hand, the animals

TABLE I.—URINE VALUES AND WATER INTAKE OF CONTROL RATS

	4 Hrs.	Std. Dev.	20 Hrs.	Std. Dev.	24 Hrs.	Std. Dev.
Volume, ml./Kg.	3.47	1.30	13.71	5.01	16.78	5.18
Na^+, micro-equiv./Kg.	149.00	81.00	1,358.00	435.00	1,501.00	351.00
Cl^-, micro-equiv./Kg.	185.00	78.00	1,049.00	234.00	1,214.00	244.00
pH	6.24	0.28	6.48	0.30	...	...
Water intake, ml./Kg.	...	...	35.8	13.6	...	...

Drinking water *ad libitum* and water dose given intraperitoneally, 0.5 ml. per 100 Gm. rat.

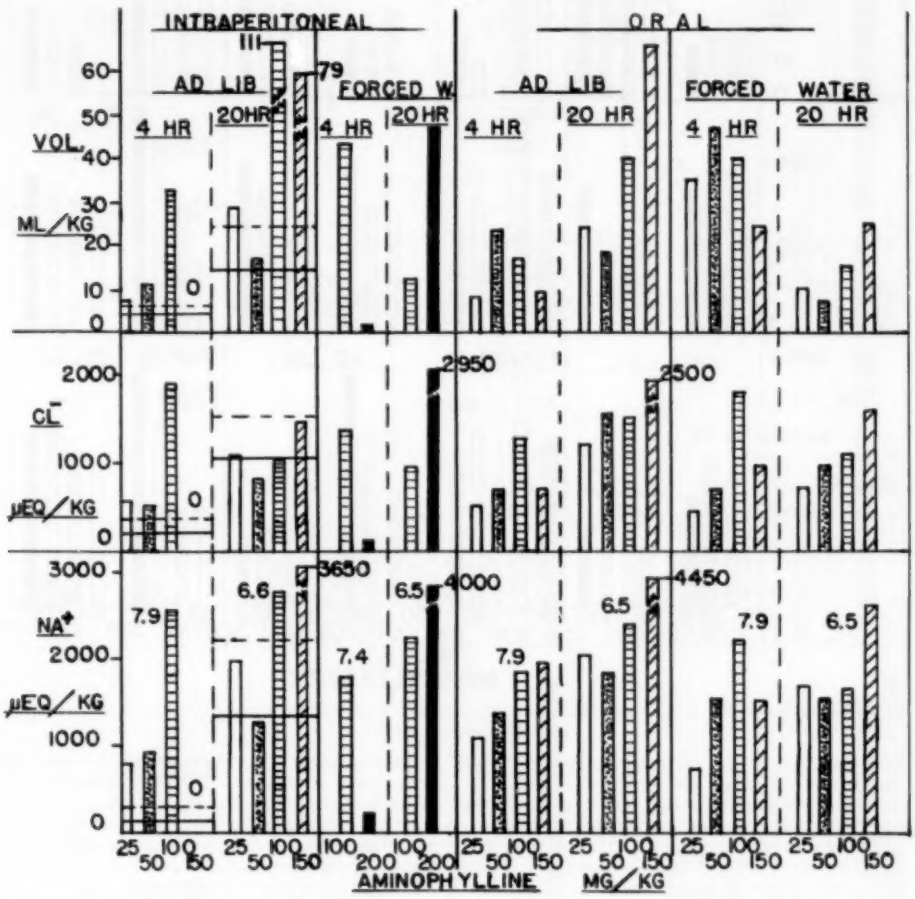

Fig. 2.—Comparison of results obtained with aminophylline given orally and intraperitoneally, with and without hydration of animals.

given water *ad libitum* yielded somewhat larger amounts of chloride and volume. Both methods gave indications that sodium was possibly being retained to some extent to balance the high excretion rate of the first four hours.

The forced hydration method was further studied with aminophylline which was given orally or intraperitoneally. As shown in Fig. 2, the excretion values increased with increasing dosage until a maximum was reached and then decreased to zero in the four-hour period with the dose of 150–200 mg. given intraperitoneally. The high doses actually inhibited urine production in the four-hour period. The saluretic action was pronounced in the following tweny hours. After oral administration the limiting effect of the high doses was not marked in the four-hour period because the drug was possibly absorbed more slowly. The rats given water *ad libitum* had more saluretic action in the twenty-hour period than those forcibly hydrated. In all cases the pH never exceeded 8 even at the highest doses in the four-hour period, and the twenty-hour period values were all within the control range. Aminometradine[4] gave a similar set of results (Fig. 3); however, the saluretic action was

[4] Mictine—G. D. Searle and Co., Chicago, Ill.

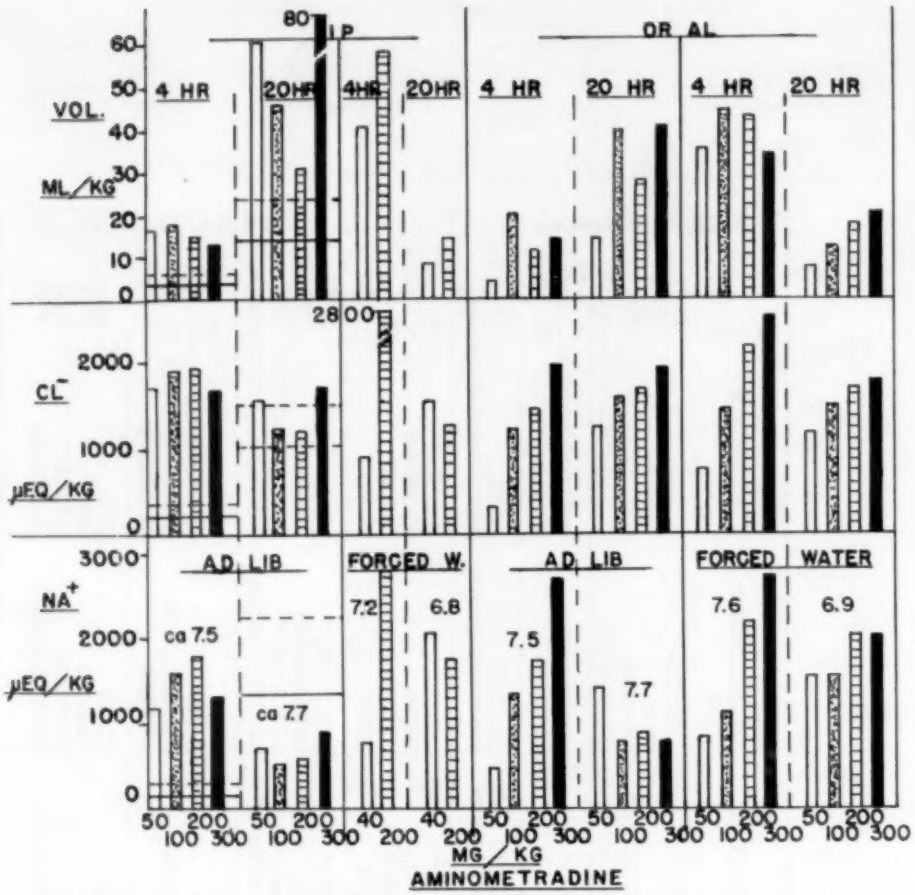

Fig. 3.—Comparison of results obtained with aminometradine given orally and intraperitoneally with and without hydration.

more apparent in the early four-hour period, although the pH values did not rise so much. In this one instance forced hydration did appear to increase the sodium output in the twenty-hour interval. Other purines studied included theophylline, xanthine, caffeine, and theobromine. These were given intraperitoneally with water *ad libitum*. As shown in Fig. 4, theophylline and caffeine exhibited saluretic action at the doses employed during the four-hour period with emphasis on volume secretion during the twenty-hour period after caffeine administration. This latter result was probably due to a thirst factor which caused the rats to drink more water. When water was withheld, very little urine was produced. In the four-hour period after theophylline the pH of the urine rose to not over 7.58 while in the case of caffeine this figure never exceeded 6.87. All other values were within the control range. Xanthine and theobromine had no activities at doses of 10 mg. or 100 mg. per Kg. weight.

Chlorothiazide[5] given intraperitoneally produced marked saluretic action during the immediate four-hour period after dosing with or without drinking water being available, see Fig. 5. Aminoisometradine[6] gave similar but possibly less striking results. However, more volume excretion was apparent in the twenty-hour period, thought to be due to the thirst factor stimulated by this drug. The pH values following chlorothiazide were around 8 after four hours, whereas after aminoisometradine they reached only 6.9.

With mercurial diuretics the dose level of mercury appears to be most critical for obtaining diuresis in the rat (5). Less than 2 mg. of mercury per Kg. rat gives little indication of diuretic activity, whereas amounts over 8 mg. per Kg. suppress urine flow. At levels of 16 mg. and 32 mg. definite damage in the rat kidney has been demonstrated histologically (8, 9). In the present study the action of chlormerodrin,[7] a mercurial urea derivative,

[5] Diuril—Merck Sharp and Dohme, Philadelphia, Pa.

[6] Rolicton—G. D. Searle and Co., Chicago, Ill.

[7] Neohydrin—Lakeside Laboratories, Inc., Milwaukee, Wis.

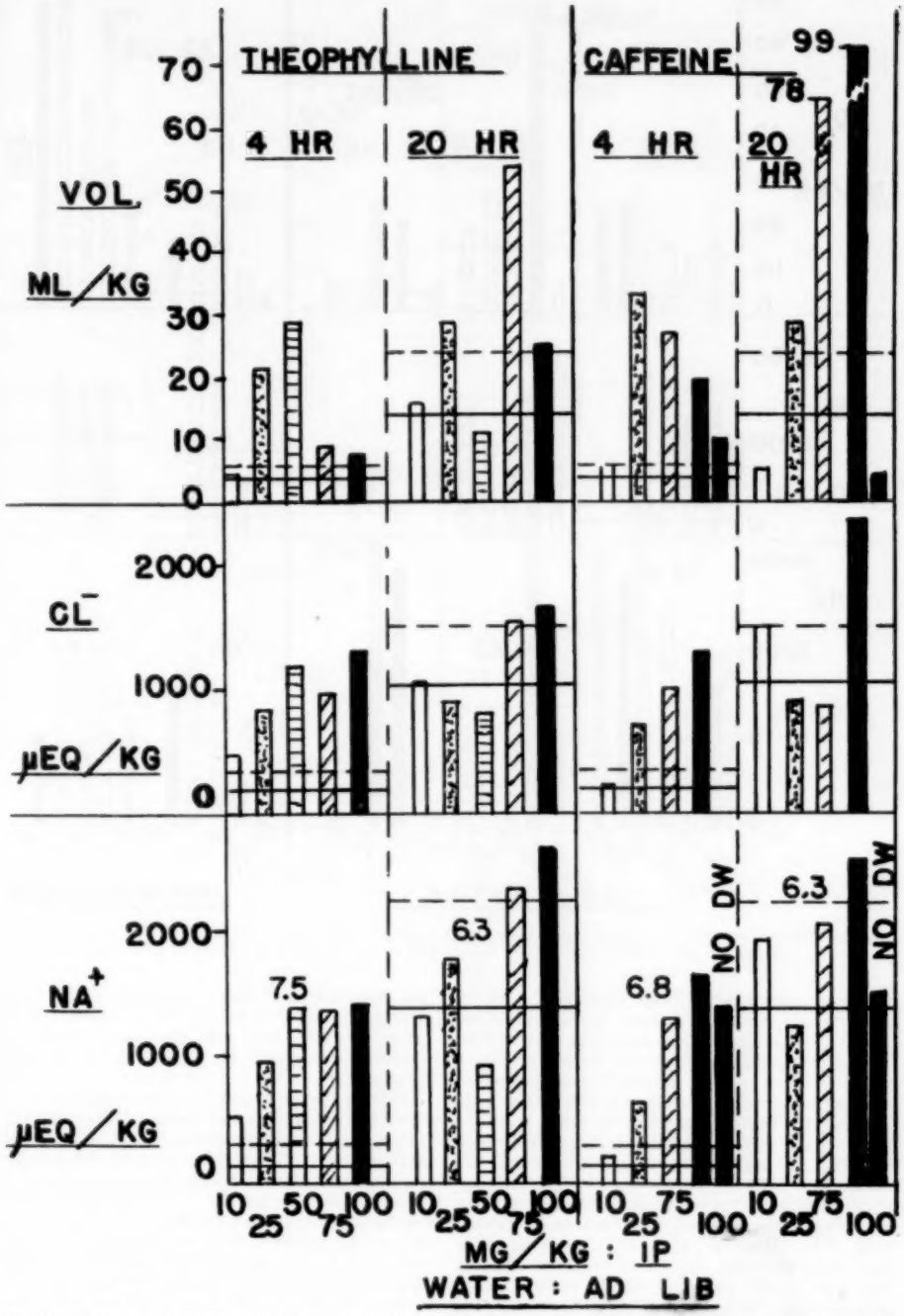

Fig. 4.—Urinary outputs produced by theophylline and caffeine given intraperitoneally to animals receiving water *ad libitum*. (No DW = no drinking water available.)

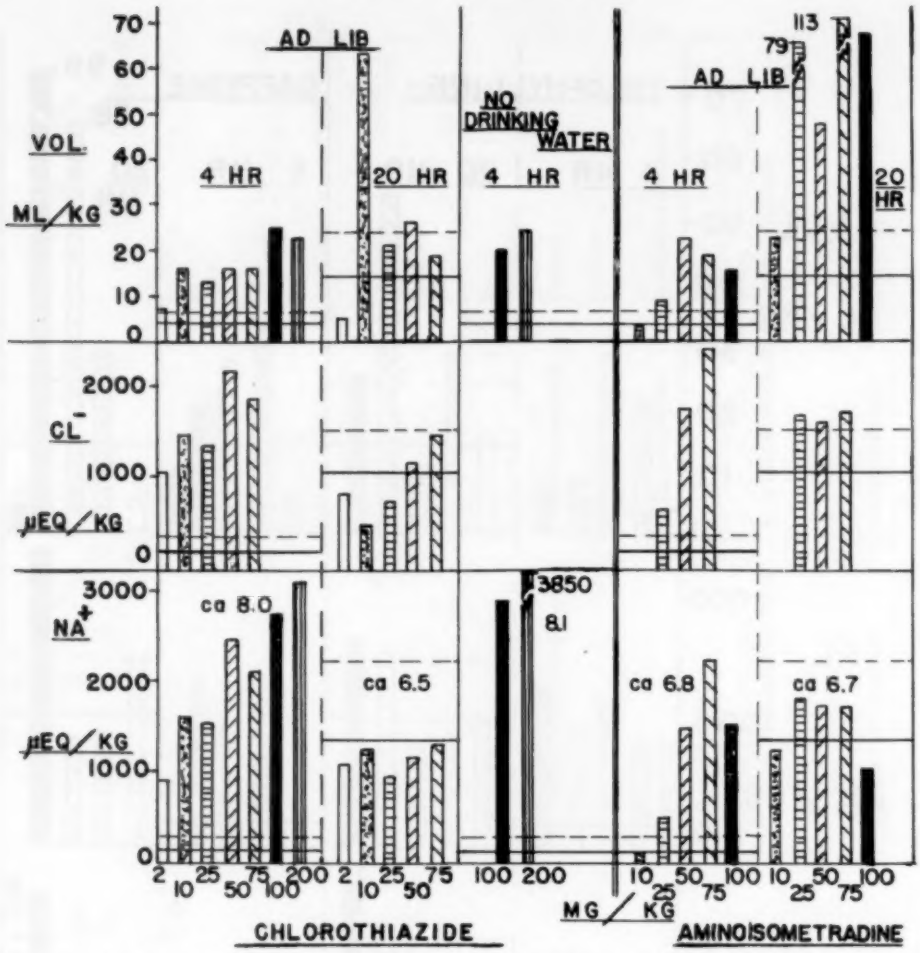

Fig. 5.—Urinary outputs obtained from chlorothiazide (with and without drinking water) and aminoisometradine given intraperitoneally to animals receiving drinking water *ad libitum*.

has been investigated. Doses containing 4 mg. of mercury per Kg. weight were injected intraperitoneally in animals which were with and without drinking water *ad libitum*. As shown in Fig. 6 there was some increase in urine volume and saluretic action in the four-hour period under both conditions. The pH values of the urine from the drug treated animals were likewise higher than their controls, 7.3 and 6.9 as compared to 6.3 and 5.9, respectively. In the twenty-hour period the mercury salt appeared to stimulate thirst when drinking water was available, with the resultant increase in urine flow. However, even when drinking water was not present the production of urine was considerably greater than that of the control group. In both cases sodium excretion was increased along with slightly higher pH values. On the other hand the chloride output seemed to be depressed.

The same dose of this mercurial given in volumes of 0.1 ml. or 2.5 ml. per 100 Gm. weight instead of the usual 0.5 ml. volume gave similar values except that in the four-hour period only, the urine volumes were somewhat less with the lower volume and slightly greater with the larger amount, as would be expected.

It is interesting to note that even with ammonium chloride the mercury salt tended to suppress chloride output as shown in Fig. 7. The acidic salt kept the pH of the urine at 6 or below. This combination caused both an increased consumption of water and a pronounced volume of urine. Other combinations with the mercury salt likewise gave interesting results. With acetazolamide the chloride output was minimized, but sodium excretion was intensified. Water intake remained below the urine volume output. The pH was elevated in both the four-hour and twenty-hour periods. On the other hand, chlorothiazide increased chloride values along with the sodium but did not change the pH to any marked degree. With aminoiso-

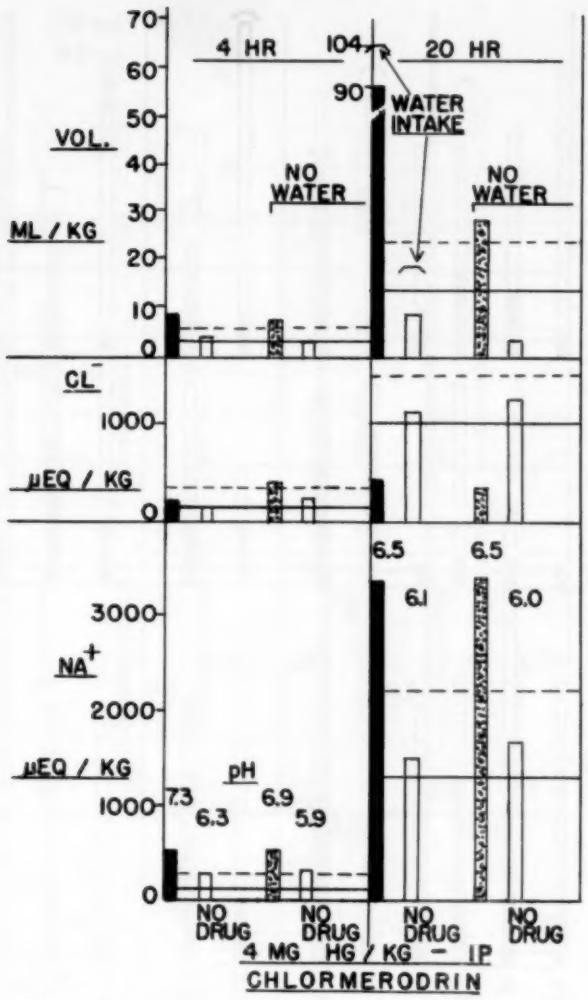

Fig. 6.—Urinary outputs obtained from chlormerodrin given intraperitoneally with different amounts of water at a dose level of 4 mg. per Kg. of mercury.

metradine the twenty-hour volume was enhanced along with water intake. Chloride amounts also rose, and the pH in the four-hour period was slightly elevated. With 8-chlorotheophylline the water intake, urine output, and sodium excretion were all enhanced in the twenty-hour period. However, the pH values did not change to any extent.

Combinations of nonmercurial diuretics with aminoisometradine are illustrated in Fig. 8. The addition of acetazolamide enhanced the sodium output and pH values but not necessarily volume which was magnified with chlorothiazide. The urine chloride was definitely increased with 8-chlorotheophylline. The latter compound also stimulated the chloride excretion when added to chlorothiazide. Acetazolamide and chlorothiazide together increased the four-hour sodium output along with higher pH values similar to the action of the carbonic anhydrase inhibitor alone.

Results from different dose levels and combinations of acetazolamide and 8-chlorotheophylline are shown in Fig. 9. At low dose levels these appeared to potentiate each other in liberating sodium in the four-hour period. Other experiments not recorded here with 5 mg. per Kg. doses of acetazolamide indicated that added caffeine, 25 mg. per Kg., had no appreciable effect on the usual result. Added triamcinolone[8] 0.5 mg. per Kg., appeared to increase sodium excretion to a slight extent. Such synthetic corticosteroids have been reported to be antagonists of aldosterone, a possible antidiuretic

[8] Aristocort—Lederle Laboratories Div., American Cyanamid Co., Pearl River, N. Y.

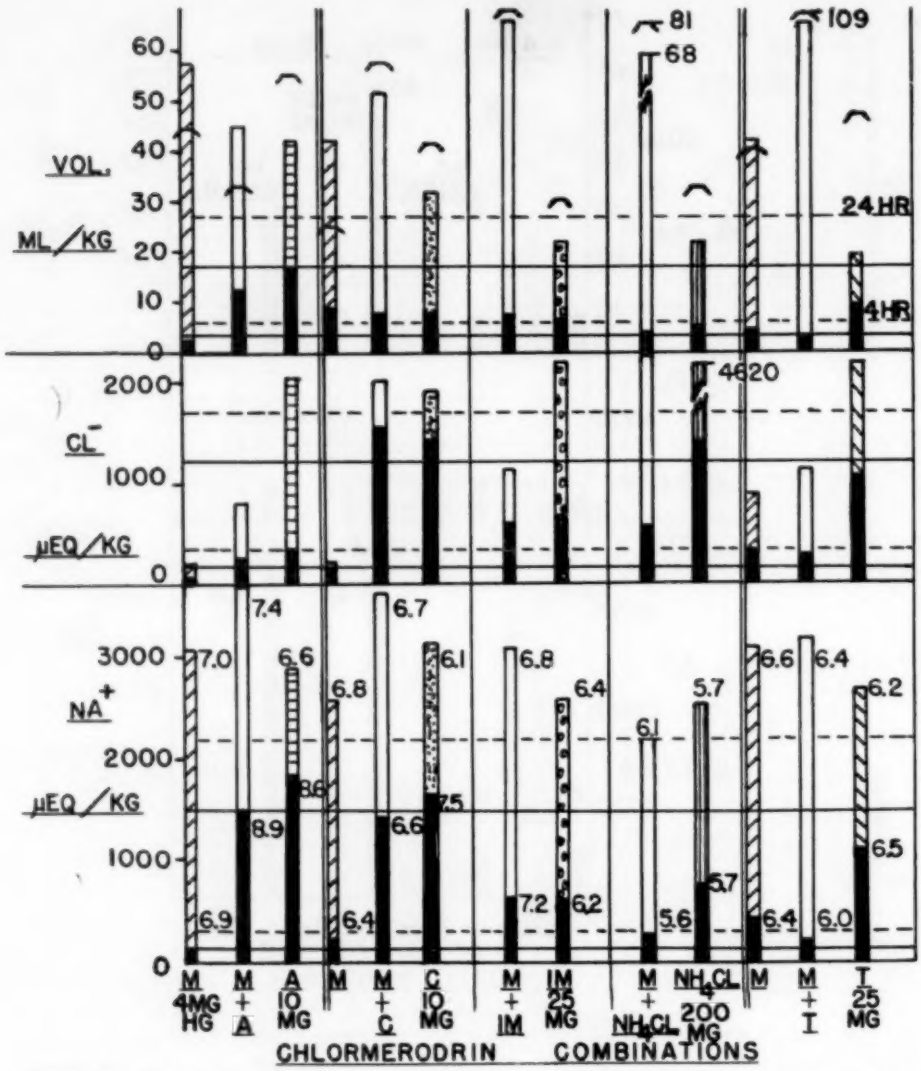

Fig. 7.—Results produced by combinations of chlormerodrin, 4 mg. of mercury per Kg. weight, with non-mercurial diuretics given intraperitoneally to rats receiving drinking water *ad libitum*. Horizontal brackets indicate volume of water consumed in the twenty-hour period. The lower two horizontal lines, solid and broken, in each section are averages for control values and upper fiducial limit (2 × S. D.) for four-hour period; the upper pair are for the twenty-hour period. M (chlormerodrin) + A (acetazolamide), 10 mg. per Kg. M (chlormerodrin) + C (chlorothiazide), 10 mg. per Kg. M (chlormerodrin) + IM (aminoisometradine), 25 mg. per Kg. M (chlormerodrin) + NH_4Cl, 200 mg. per Kg. M (chlormerodrin) + T (8-chlorotheophylline), 25 mg. per Kg.

hormone (10). Theophylline, 25 mg. per Kg., with acetazolamide increased sodium along with volume output and water consumption. Ammonium chloride, 200 mg. per Kg., intensified the chloride output and slightly lowered the pH values, as would be expected.

Various compounds have been screened for diuretic activity by the above procedure. These included purines, pyrimidines, pyrrolopyrimidines, triazines, and other miscellaneous types. Examples are given in Table II. Due to either toxicity or inactivity at low dosages none of these were considered useful diuretics in themselves. However, the activity of low doses of acetazolamide, 5 mg. or 10 mg. per Kg., was markedly enhanced by the coadministration of 10 mg. or 20 mg. per Kg. of 4-(sec-butylamino)-pyrrolo-[2,3-*d*]-pyrimidine (B.W. 57–10).

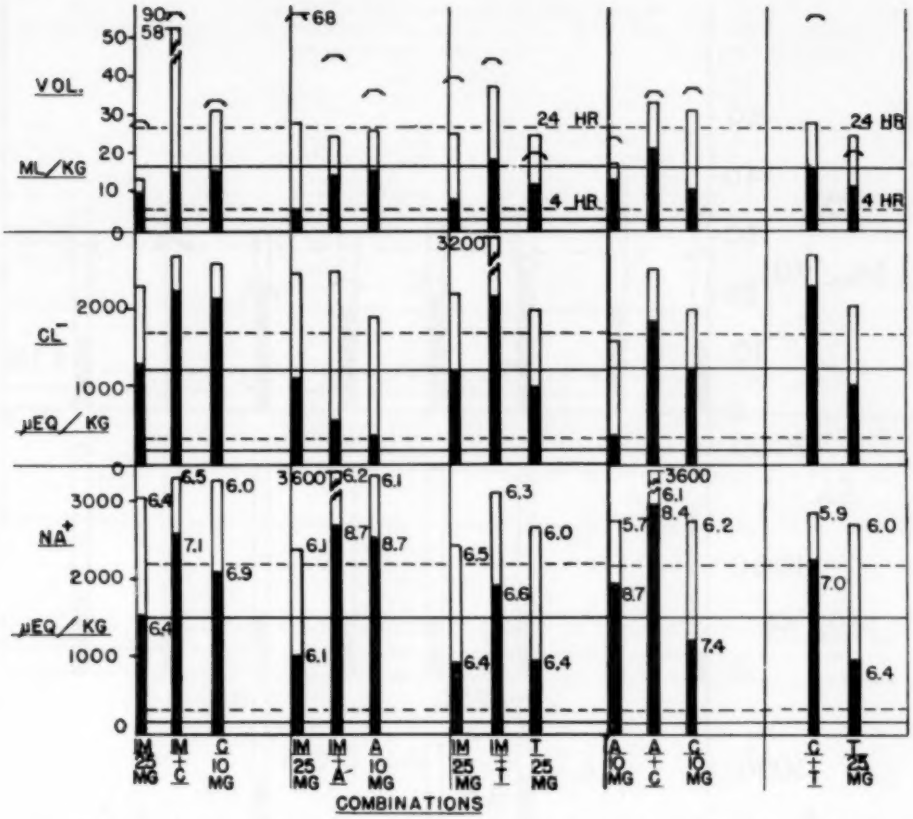

Fig. 8.—Results produced with combinations of diuretics given intraperitoneally with water *ad libitum*. IM (aminoisometradine), 25 mg. per Kg., + C (chlorothiazide), 10 mg. per Kg. IM (aminoisometradine), 25 mg. per Kg., + A (acetazolamide), 10 mg. per Kg. IM (aminoisometradine), 25 mg. per Kg., + T (8-chlorotheophylline), 25 mg. per Kg. A (acetazolamide), 10 mg. per Kg., + C (chlorothiazide), 10 mg. per Kg. C (chlorothiazide), 10 mg. per Kg., + T (8-chlorotheophylline), 25 mg. per Kg.

To enhance the activity of chlormerodrin it was theorized that brombenzene (11) would reduce the amount of —SH groups available to react with the mercury compound and thus enable the diuretic to become more effective (12). However, when rats were fed a diet containing 4% brombenzene or given 100 mg. per Kg. daily by stomach tube, the diuretic activity of the mercurial was not increased. A dose of *n*-ethyl-maleimide, 1 mg. per Kg. orally, likewise did not assist activity.

Since male animals had been found to give somewhat better indications of diuretic activity (5), it was believed that treatment of males or females with the opposite sex hormones might intensify either diuresis or nondiuresis. Therefore, recently weaned rats of both sexes were given diets providing 500 μg. of methyltestosterone or 50 μg. of estradiol per animal per day for six weeks before diuretic testing. The results gave only slight suggestions that the female hormone decreased diuresis in both sexes when treated with acetazolamide, aminoisometradine, or chlormerodrin.

SUMMARY

Patterns of urinary excretion produced by diuretics administered to both excessively hydrated and untreated rats have been described. Excessive hydration was found to be unnecessary. The diuretic actions found could be divided into two categories; carbonic anhydrase inhibitors (acetazolamide), which rapidly increased the urine volume and sodium excretion with a high pH, and saluretic agents: (*a*) Pyrimidine (aminoisometradine), purine (aminophylline), and chlorothiazide types which also rapidly increased the volume with saluretic activity but not elevating the pH to the above extent. (*b*) Mercurials (chlormerodrin) which had little immediate activity but produced a delayed urine output with somewhat depressed chloride values. (*c*) Mis-

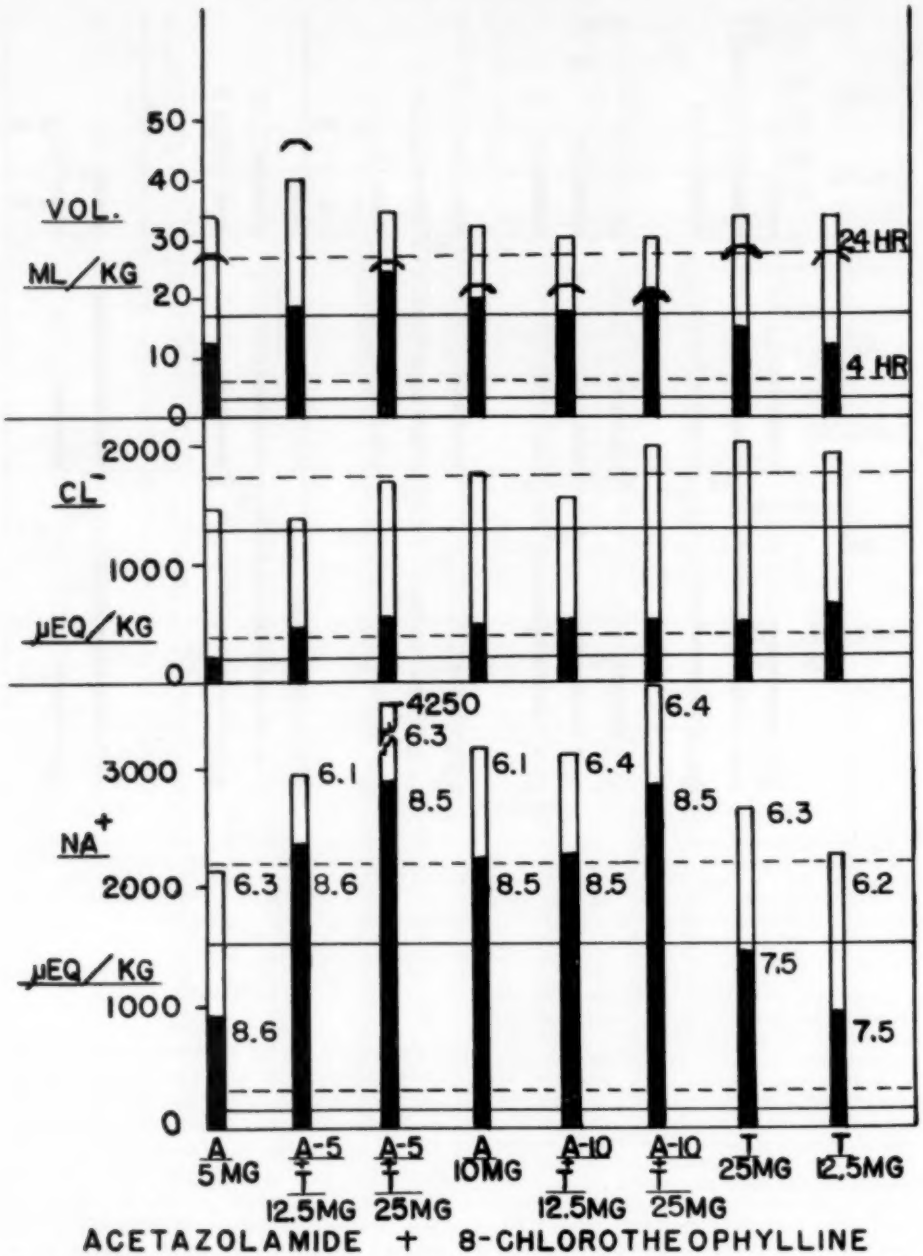

Fig. 9.—Results produced with varied combinations of acetazolamide (A) and 8-chlorotheophylline (T).

cellaneous types (triazines, etc.) which had immediate saluretic activity within narrow dose ranges. Combinations of these proved interesting with possibly some potentiation of activities observed, especially with acetazolamide and 8-chlorotheophylline. Due to the inactivity of —SH binding agents in enhancing the diuretic activity of chlormoren driit is suggested that this mode of action of mercurials be considered as still open to question.

TABLE II.—EXAMPLES OF COMPOUNDS TESTED[a]

	Urine Totals for 4 Hours					Urine Totals for 20 Hours			
	Dose, mg./Kg.	Volume, ml./Kg.	Na^+, micro-equiv./Kg.	Cl^-, micro-equiv./Kg.	pH	Volume, ml./Kg.	Na^+, micro-equiv./Kg.	Cl^-, micro-equiv./Kg.	pH
Control,	...	3.47	149	185	6.24	13.71	1,358	1,049	6.48
B. W. 49-335, 2,8-dichloro-6-aminopurine	10	7.76	568	552	5.93	33.2	1,780	1,220	6.61
	100	18.88	1,408	1,648	6.52	107.2	5,580	4,040	6.62
B. W. 57-10, 4-(sec-butylamino)-pyrrolo-[2,3-δ]-pyrimidine	10	8.00	272	236	6.65	51.8	2,640	1,640	6.71
	100	17.40	1,048	388	7.47	14.4	1,600	1,440	6.12
B. W. 55-325, 2-amino-4-hydroxy-5-methyl-6-phenyl-pyrimidine	25	8.08	364	...	7.12	43.4	1,300	...	6.86
	50	10.76	1,020	...	6.18	65.6	1,860	...	6.66
	250[b]	1.24	68	...	5.66	34.6	3,940	...	6.51
B. W. 53-309, N-phenyl-N′-acetylurea	10	9.72	856	720	6.29	77.8	2,040	1,460	6.39
	100		no urine			57.8	9,240	5,320	6.31
B. W. 57-205, 2-amino-4-(2,4-dichloro-anilino)-1,3,5-triazine · HCl	2.5	7.72	440	572	6.17	23.0	2,020	1,560	6.62
	5.0	10.40	736	752	6.17	29.6	1,880	1,160	6.45
	10.0	8.52	708	708	6.65	20.8	1,900	1,360	6.28

[a] These compounds were supplied by the Chemotherapeutic Section of the Wellcome Research Laboratories, Tuckahoe, N. Y. [b] One rat died at this dose.

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Salts of *p*-Acetamidobenzoic Acid*

By ANDREW LASSLO, CARL C. PFEIFFER, and PAULINE D. WALLER

p-Acetamidobenzoic acid yields atmospherically stable salts with amines which have a tendency to give hygroscopic products with conventionally used acid components. The physical properties of p-acetamidobenzoic acid salts, combined with the innocuous nature and absorption characteristics of the acid, render the latter valuable as a salt-forming agent for pharmaceutical preparations. Procedures for the preparation of p-acetamidobenzoic acid salts are reported.

RECENTLY, Pfeiffer (1) and his co-workers reported the effect of 2-dimethylaminoethanol (DMAE) upon the central nervous system and called attention to its potentialities in the treatment of mental disorders. In connection with an extensive clinical study, we became interested in preparing a stable nonhygroscopic salt of DMAE which would facilitate adequate standardization of oral administration and permit satisfactory correlation of the data obtained by the participants in the investigation. Of several possible salts, the salicylate, *d*-tartrate, *d*-pantoate, and *p*-acetamidobenzoate were chosen for evaluation. Salicylate and tartrate salts are frequently employed in pharmaceutical preparations, *d*-pantoic acid is a component of the vitamin pantothenic acid, and *p*-acetamidobenzoic acid is the normal metabolite of *p*-aminobenzoic acid.

In a study of the physical properties of these four salts, DMAE *p*-acetamidobenzoate[1] was found to be the least hygroscopic (Table I). In regard to toxicity, one could hardly find a more

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Patent applied for.

[1] Marketed as Deaner by the Riker Laboratories, Inc., Los Angeles, Calif.

TABLE I.—COMPARATIVE EFFECT OF AVERAGE ATMOSPHERIC CONDITIONS

Salt	Weight[a] of Sample, mg.	Period of Exposure in a Nonairconditioned Room, Hours	Change in Weight mg.	Change in Weight %
2-Dimethylaminoethanol *d*-pantoate	500	64	+132.2	+26.44
2-Dimethylaminoethanol salicylate[b]	1,000	64	+154.2	+15.42
2-Dimethylaminoethanol *d*-tartrate (bitartrate)[b]	1,000	64	+57.7	+5.77
2-Dimethylaminoethanol *p*-acetamidobenzoate	500	64	−1.4	−0.28

[a] Prior to evaluation, samples were dried at 25°/0.07 mm. Hg for twenty-eight hours.
[b] Courtesy Riker Laboratories, Inc., Los Angeles, Calif.

innocuous acid component. Cantarow and Schepartz (2) report its presence in normal blood and urine, and consider it a metabolite of folic acid (for details on the metabolism of *p*-acetamidobenzoic acid in humans see Zehender's work (3)). These properties render *p*-acetamidobenzoic acid particularly valuable as a salt-forming agent for pharmaceutical preparations; especially so, when administration over an extended period of time is necessary. The efficacy of *p*-acetamidobenzoic acid acquires additional emphasis, if we consider the disadvantages one may encounter with salicylic and tartaric acids. The toxic properties of salicylic acid and the so-called "salicylates" are well known; diminished caloric intake may predispose a person to serious intoxication with no more than usual doses of "salicylates." Some asthmatic patients are sensitive to minute doses of salicylate. There is evidence (4) that salicylates may accumulate, if administered over a prolonged period of time; this becomes of particular concern, if renal function is below normal. In regard to tartaric acid salts, questions have been raised as to the nature and degree of their absorption from the human intestine (5, 6); actually several tartrates are known as cathartics, and cathartic action argues against much absorption from the intestinal tract (7). More specifically, Harington (8) states that orally administered hexamethonium bitartrate was always more poorly absorbed than other orally administered hexamethonium salts. In accordance with these findings, Pfeiffer (9) also observed a substantial decrease in the effectiveness of orally administered DMAE when given in the form of its *d*-bitartrate. It is interesting to note that Hagen (10) reported loss of *l*-epinephrine activity in stimulating oxygen consumption of mouse parotid glands incubated *in vitro* in a calcium-free medium, when the base was administered as the *d*-bitartrate salt.

The desirable properties of DMAE *p*-acetamidobenzoate suggested the use of *p*-acetamidobenzoic acid in the preparation of salts of other bases which have a tendency to yield hygroscopic salts with conventionally used acid components. The new salts are listed in Table II.

EXPERIMENTAL

The compounds listed in Table II were prepared by the following procedures:

Procedure A.—2-Dimethylaminoethanol *p*-Acetamidobenzoate.—Forty grams (0.223 mole) of *p*-acetamidobenzoic acid was dissolved in 500 ml. of boiling absolute methanol. Heating was discontinued, and a solution of 19.9 Gm. (0.223 mole) of dimethylaminoethanol in 100 ml. of absolute methanol was added with mechanical stirring, as fast as the exothermic nature of the reaction permitted. The resulting solution was allowed to cool to room temperature and was suction-filtered through Celite (Johns-Manville filter aid). The filtrate was poured into 500 ml. of anhydrous ethyl ether seeded[1] with a few crystals of DMAE *p*-acetamidobenzoate; crystallization ensued immediately. The product, a white crystalline material, was dried at 25°/0.08 mm. Hg for fifteen hours (48.4 Gm., 80.9% yield). It may be recrystallized from absolute ethanol–ethyl acetate, and dried as indicated above. The salt is readily soluble in water and is very stable over a wide range of atmospheric conditions. During its characterization, we have found that it will decompose when exposed to high temperatures under vacuum (100°/0.08 mm. Hg) and yield free *p*-acetamidobenzoic acid (72.5 mg. of the salt yielded quantitatively 47.8 mg. of the acid after four hours at 100°/0.08 mm. Hg).

Choline *p*-acetamidobenzoate remained unaffected under the same conditions; this was probably due to the increased basicity of choline.

Procedure B.—Nicotine *p*-Acetamidobenzoate.—Twenty grams (0.112 mole) of *p*-acetamidobenzoic acid was dissolved in 300 ml. of boiling absolute methanol. Heating was discontinued and 20.0 Gm. (0.124 mole) of nicotine was added. The resulting solution was thoroughly mixed, suction-filtered through Celite, and the solvent was removed under reduced pressure (max. pot temp. 50°). The viscous residue was mechanically agitated in 200 ml. anhydrous ethyl ether; it gradually turned into a pink powder. The crude product was dried at 25°/0.1 mm. Hg for one hour (32.2 Gm., 84.5% yield). Upon recrystallization from absolute ethanol–ethyl acetate, the pure salt was obtained in the form of pink crystals, after a first crop of unreacted *p*-acetamidobenzoic acid; it was dried as indicated above. The salt is readily soluble in water and is

[1] Three to six milliliters of filtrate added to 10 ml. of anhydrous ethyl ether yielded crystals within ten to fifteen minutes at room temperature.

TABLE II.—PHYSICAL AND ANALYTICAL DATA

Compound	Method of Preparation	Melting Point, °C.[a]	Optical Properties	Empirical Formula	Analyses[b] C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	N, % Calcd.	N, % Found
2-Dimethylaminoethanol *d*-pantoate	C	...	n_D^{27} 1.4757 $[\alpha]_D^{28}$ +12.9° (c = 13.4, in abs. methanol)	$C_{13}H_{25}NO_5$	50.61	50.68	9.77	9.51	5.90	6.06
2-Dimethylaminoethanol *p*-acetamidobenzoate	A	159.0–161.5	...	$C_{13}H_{20}N_2O_4$	58.19	58.20	7.51	7.64	10.44	10.5
2-Diethylaminoethanol *p*-acetamidobenzoate	B	129.0–131.0	...	$C_{15}H_{24}N_2O_4$	60.78	60.79	8.16	8.21	9.46	9.46
Triethylamine *p*-acetamidobenzoate	B	137.0–138.0	...	$C_{15}H_{24}N_2O_3$	64.25	64.10	8.63	8.55	10.00	9.93
Choline *p*-acetamidobenzoate	A	231.6–232.2	...	$C_{14}H_{22}N_2O_4$	59.55	59.56	7.86	7.76	9.93	9.68
Nicotine *p*-acetamidobenzoate	B	Decomp. at 134–137	$[\alpha]_D^{25}$ +14.0° (c = 8, in H_2O)	$C_{15}H_{15}N_3O_3$	66.84	66.96	6.79	6.86	12.31	12.15

[a] Uncorrected. [b] Analyses by Drs. G. Weiler and F. B. Strauss, Oxford, England.

stable under atmospheric conditions; it will decompose when exposed to high temperatures under vacuum (100°/0.1 mm. Hg).

Procedure C.—2-Dimethylaminoethanol *d*-Pantoate.—To an aqueous solution (50 ml.) of *l*-pantoyl lactone (5.07 Gm., 0.039 mole), 4.89 Gm. (0.028 mole) of barium hydroxide was added and the resulting solution was heated at 74–81° for three hours. Subsequently, excess barium was precipitated as the carbonate by bubbling a stream of carbon dioxide through the hot solution, and the precipitate was filtered off with Celite. The filtrate, containing 0.019 mole of barium pantoate, was cooled to room temperature and added to an aqueous solution (26 ml.) of 0.019 mole of dimethylaminoethanol sulfate (3.39 Gm. of dimethylaminoethanol neutralized with sulfuric acid to pH 6.8). The reaction mixture was thoroughly mixed and allowed to stand overnight. The precipitated barium sulfate was filtered off with Celite, and the water was removed under reduced pressure (max. pot temp. 30°). The viscous residue was dissolved in about 300 ml. of absolute methanol, the solution was filtered through Celite, and the solvent was removed under reduced pressure. The residue was redissolved in another 300 ml. of absolute methanol, the solution was refrigerated for about fifty hours, filtered through Celite, the solvent was removed under reduced pressure, and the residue was subjected to a vacuum of 0.07 mm. Hg at 25° for a period of forty-three hours. The product (7.5 Gm., 83.2% yield) was a clear, yellow, viscous liquid, which has retained its original consistency over a period of ten months; all attempts to crystallize the salt proved unsuccessful.

SUMMARY

The suitability of nonhygroscopic *p*-acetamidobenzoic acid salts for pharmaceutical preparations was pointed out. The preparation of the *p*-acetamidobenzoic acid salts of nicotine, choline, 2-dimethylaminoethanol (DMAE), 2-diethylaminoethanol, and triethylamine, and that of the *d*-pantoic acid salt of DMAE was described.

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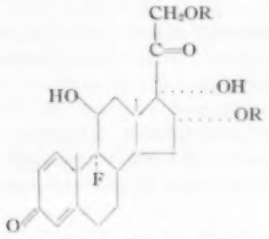
16α-Hydroxy Steroids I*

Characterization of Triamcinolone

By LELAND L. SMITH and MURRAY HALWER

Triamcinolone and triamcinolone 16α,21-diacetate have been characterized by ultraviolet absorption spectra, infrared absorption spectra, absorption spectra in sulfuric acid, polarography, reduction of tetrazolium blue, and by Porter-Silber chromogens. The use of absorption spectra, polarography, and colorimetry for quantitative determination of bulk triamcinolone and of triamcinolone in tablets is discussed. Some new chemistry of 16α-hydroxylated steroids is reviewed.

TRIAMCINOLONE (9α - fluoro - 11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione) is a new potent corticosteroid of clinical importance (1–3). Triamcinolone is currently marketed in this country as the alcohol, although the 16α,21-diacetate has been tested clinically (4). Characterization of the hormone has been limited to brief accounts in the chemical journals (5–7) and in the patent literature (8). A more detailed characterization of both triamcinolone and triamcinolone diacetate is the subject of this report.



I. R = H II. R = $COCH_3$

Triamcinolone (I) and its diacetate (II) are distinguished structurally from other commercially important corticosteroids by the presence of the 16α-hydroxyl group, the $\Delta^{1,4}$-3-ketone, 11β-hydroxyl,9α-fluoro, and dihydroxyacetone side-chain features being common to other hormones. The profound biological effect of the 16α-hydroxyl group in the steroid molecule is accompanied by some altered physical and chemical properties, as certain typical reactions of corticosteroids are influenced by the introduction of the 16α-hydroxyl.

Absorption spectra in alcohol and in alcoholic alkali (9) (Tables III and IV) and in sulfuric acid (8, 10) (Table V), characteristic polarographic reduction behavior (11, 12), and infrared absorption spectra (Figs. 1 and 2) in the 6.0–6.25 μ region (11, 13) exhibited by triamcinolone and by its diacetate are typical of $\Delta^{1,4}$-3-ketosteroids of this series. Triamcinolone forms a diacetate (5) and a bis-2,4-dinitrophenylhydrazone (8). The usual color tests (spot tests on filter paper) used for the detection of Δ^4-3-ketosteroids, such as Bush's alkaline fluorescence (14), 2,4-dinitrophenylhydrazine (15), *p*-phenylenediamine phthalate (16), etc., fail with triamcinolone as they do with other $\Delta^{1,4}$-3-ketones. Isonicotinic acid hydrazide has proved of use in detection of triamcinolone (17), the reported failure of the reagent for detecting other $\Delta^{1,4}$-3-ketosteroids notwithstanding (18). Triamcinolone fails to react with Girard P reagent in the usual manner, such behavior again being expected of $\Delta^{1,4}$-3-ketones (19). This failure to react with Girard P reagent permits separation of triamcincolone from related Δ^4-3-ketones.

The presence of the 16α-hydroxyl group adjacent to the dihydroxyacetone side-chain affects the response of the steroid in several analytical procedures and enables one to prepare derivatives characteristic of planar *cis*-diols. Thus 16α,17α-acetonides (7, 20) and 16α,17α-cyclic borates (21) have been reported. Although triamcinolone has the anticipated reducing action toward tetrazolium blue on spot testing on filter paper, instrumentally the response to tetrazolium blue (22) is about 70% greater than that for non-16α-hydroxylated analogs. This property is suggested as characteristic of 16α,17α,21-trihydroxy-20-ketones (*cf.* Table I). The cyclic 16α,17α-diol derivatives respond more like the non-16α-hydroxylated parent steroids, the 16α,17α-acetonide of triamcinolone having but 40% of the response of triamcinolone, the 16α,17α-cyclic borate preparations having 60–65% of the response.

Triamcinolone does not respond to the Porter-Silber test (23) in the anticipated manner (*cf.* Table II). The response of triamcinolone and a variety of other 16α,17α,21-trihydroxy-20-ketones is depressed to about 10% of that of the non-16α-hydroxyl analogs. This poor response

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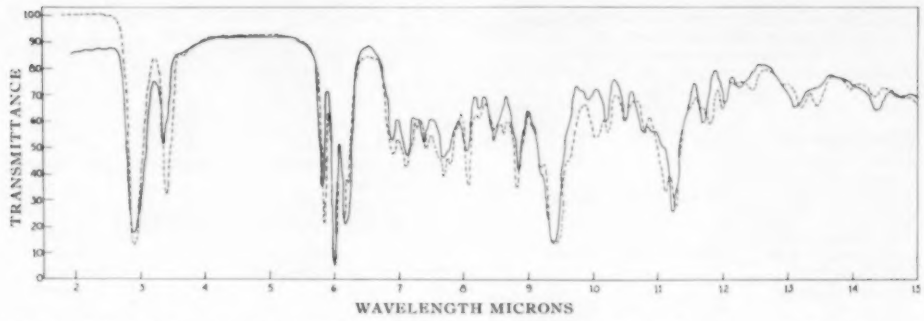

Fig. 1.—Infrared absorption spectra of triamcinolone polymorphs in KBr disks. ———— Type I, - - - - Type II.

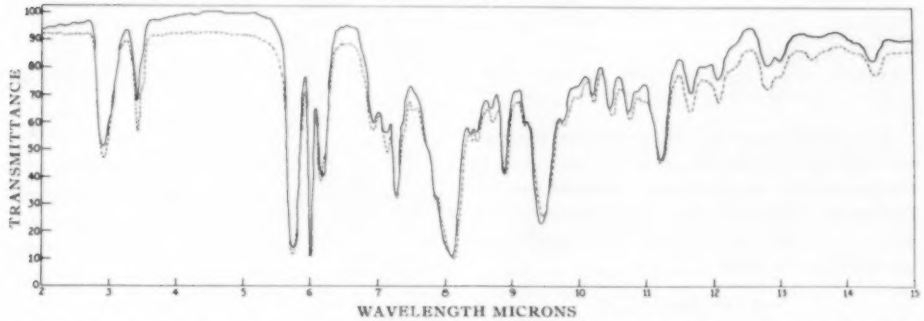

Fig. 2.—Infrared absorption spectra of triamcinolone 16α,21-diacetate polymorphs in KBr disks. ———— Type I, - - - - Type II.

obviates the use of the Porter-Silber assay in biological systems where non-16α-hydroxylated steroids are also to be measured.[1]

Triamcinolone and the related 1,2-dihydro compound (9α-fluoro-11β,16α,17α,21-tetrahydroxy-4-pregnene-3,20-dione) fail to give positive tests for the 17α-hydroxyl group with the sulfuric acid-phosphorus pentoxide reagent of Steyermark and Nowaczynski (24). No selective absorption occurs in the 400–600 mμ region. The generality of this test must remain questionable on other grounds, as neither prednisone, prednisolone, nor 9α-fluoroprednisolone give selective absorption in the 400–600 mμ region. Since 9α-fluorohydrocortisone does have selective absorption at *ca.* 550 mμ (E 1%, 1 cm. 159), both the 1,2-double bond and the 16α-hydroxyl may interfere with this color test.

Triamcinolone isomerizes under a variety of conditions to form a tetrahydroxy-diketone; such isomerization is also observed for at least one other 16α-hydroxylated steroid, 9α-fluoro-11β,16α,17α,21-tetrahydroxy-4-pregnene-3,20-dione. These isomers are more polar than the parent steroid, and may be expected to interfere with certain instrumental assays for triamcinolone. The isomerization of triamcinolone will be the subject of a later report.

TABLE I.—RELATIVE REDUCING POWER WITH TETRAZOLIUM BLUE OF 16α-HYDROXYLATED STEROIDS

Parent Steroid	Reducing Power of Parent Non-16α-Hydroxylated Steroids $E^{1\%}_{1\text{ cm.}}$ 510–530 mμ	Reducing Power of 16α-Hydroxylated Analog $E^{1\%}_{1\text{ cm.}}$ 510–530 mμ
11β,17α,21-Trihydroxy-4-pregnene-3,20-dione (hydrocortisone)	610	1,050
9α-Fluoro-11β,17α,21-trihydroxy-4-pregnene-3,20-dione (9α-fluorohydrocortisone)	610	1,040
11β,17α,21-Trihydroxy-1,4-pregnadiene-3,20-dione (prednisolone)	640	1,060
9α-Fluoro-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione (9α-fluoroprednisolone)	620	1,050

The tetrazolium blue determinations were carried out as described in the *Experimental* part, read *vs.* a reagent blank after thirty minutes standing at room temperature.

[1] The failure of triamcinolone to undergo the Porter-Silber reaction has recently been reported: Walker, A. and Schlunke, H. P., *Experientia*, **15**, 71(1959). The increased reducing power of triamcinolone towards alkaline ferricyanide, as compared with non-16 α-hydroxylated steroids has also been noted: Stephenson, N. R., *Can. J. Biochem. and Physiol.*, **37**, 391(1959).

TABLE II.—RESPONSE TO PORTER-SILBER CONDITIONS OF TRIAMCINOLONE AND RELATED 16α-HYDROXYLATED STEROIDS

Parent Steroid Nucleus	Non-16α-Hydroxylated Steroid $\lambda_{max.}$	Non-16α-Hydroxylated Steroid $E^{1\%}_{1\ cm.}$	16α-Hydroxylated Steroid $\lambda_{max.}$	16α-Hydroxylated Steroid $E^{1\%}_{1\ cm.}$
9α-Fluoro-11β,17α,21-trihydroxy-4-pregnene-3,20-dione	417 mμ	1,350	420 mμ	241
9α-Fluoro-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione	416 mμ	1,800	425 mμ	165
11β,17α,21-Trihydroxy-4-pregnene-3,20-dione	415 mμ	1,650	415 mμ	191
11β,17α,21-Trihydroxy-1,4-pregnadiene-3,20-dione	415 mμ	1,990	420 mμ (Inflection)	238
17α,21-Dihydroxy-4,9(11)-pregnadiene-3,20-dione	415 mμ	1,785	415 mμ	114

Analytical techniques which involve differential migration of the steroid will be described in the second paper of this series. Some useful procedures for identification of triamcinolone and for analysis of bulk and tableted triamcinolone are presented in this paper.

TRIAMCINOLONE

Identification.—Triamcinolone is characterized by the physical measurements of Table III. Triamcinolone may exist in at least two polymorphs, depending on the solvent used for crystallization, such behavior rendering the melting point of little use in identification. Characteristic infrared spectra of triamcinolone in the solid state serve to distinguish the polymorphs one from the other. Type I polymorph (obtained from pyridine, aqueous pyridine, etc.) and type II polymorph (from methanol or aqeuous methanol) are interconvertible. Whereas the usual infrared bands characteristic of $\Delta^{1,4}$-3-ketones (11, 13) are present in both types, the type I spectra does not differentiate the Δ^1- and Δ^4-bands, the two bands coalescing into one broad band at 6.18 μ, whereas type II spectra give well defined bands at 6.15 μ and 6.22 μ (Fig. 1). The fingerprint region also contains select points of difference. Mixed spectra (containing aspects of both type I and type II spectra) have been observed in some samples.

Although infrared identification of triamcinolone is preferable, absorption spectra in concentrated sulfuric acid may be used; the band positions at 260–263 mμ and at 310 mμ (Table V) distinguish triamcinolone from a host of other related steroids (10).

Of particular value is the distinctive time course of alteration of the sulfuric acid spectra. The steady increase in absorbance of the 375–390 mμ band together with its shift from 390 mμ to 375 mμ over twenty hours is not duplicated even by triamcinolone 16α,21-diacetate.

Determination.—Instrumental determination of triamcinolone may be made with a variety of techniques depending on the specificity required. Direct ultraviolet absorption measurements, direct polarographic measurements, and tetrazolium blue reducing power have served most uses. A preferred method combines polarographic determination (ring-A) together with determination with tetrazolium blue (side-chain). Where more specificity is required, chromatographic separations must precede instrumental determinations.

TABLE III. PHYSICAL PROPERTIES OF TRIAMCINOLONE

Property	Bernstein, *et al.*, *J. Am. Chem. Soc.*, **78**, 5693 (1956).	Thoma, *et al.*, *J. Am. Chem. Soc.*, **79**, 4818 (1957).	This Report
Melting Point (capillary)	260–262.5°[a]	248–250°	262–264°dec.[b]
Specific Rotation	$[\alpha]_D^{23}$ + 75° (acetone)	$[\alpha]_D^{23}$ + 71° (acetone)	$[\alpha]_D^{23}$ + 67.1° ±1.9° (MeOH)
EtOH (ε) $\lambda_{max.}$	238 mμ. (15,800)	....	239 mμ. (15,224 ± 122)[c,d]
Infrared Absorption	$\lambda_{max.}^{KBr}$ 3,388, 1,705, 1,660, 1,620, 1,604 cm.$^{-1}$	$\lambda_{max.}^{Nujol}$ 2.95, 5.85, 6.02, 6.16, 6.24, 11.26 μ	$\lambda_{max.}^{KBr}$ 2.95, 3.41, 3.45, 5.85, 6.01, 6.15, 6.21, 11.23μ
0.066 *N* NaOH in EtOH (ε) $\lambda_{max.}$	....	....	240 mμ (14,500), 264 mμ (7,800 inflection), 310 mμ (2,270)[e]

[a] Bernstein, *et al.*, also report a sample of triamcinolone melting at 269–271°.
[b] Some preparations of triamcinolone have been observed to melt as high as 273–275° decompn.
[c] An average of six determinations with standard deviation.
[d] An inflection at 308 mμ (ε89) may be seen at high steroid concentration.
[e] Spectra taken three to five minutes after solution at room temperature. At 60° for one hour, 239 mμ (14,900), 264 mμ (7,800, inflection), 310 mμ (3,480), not changed after two further hours at 60°.

Triamcinolone Purification.—A selected, repeatedly recrystallized batch of triamcinolone, m. p. 249–250°; $[\alpha]_D^{23}$ + 67.4° (MeOH); $\lambda_{max.}^{EtOH}$ 239 mμ (ε 14,900); was partitioned on a column prepared as follows: 600 Gm. of acid-washed Celite diatomaceous earth was moistened with 300 ml. of the lower phase of the mixture: dioxane (10 liters) : cyclohexane (6 liters): water (2 liters), then packed in 2–3 inch layers into a three-inch diameter column. The sample of 600 mg. of triamcinolone dissolved in 20 ml. of lower phase was slurried into 40 Gm. of Celite diatomaceous earth and the charge packed onto the column. Development of the column with the upper phase of the solvent mixture eluted triamcinolone at 6.2–6.9 hold-back volumes (HBV) (1 HBV was 900 ml.). The fraction was evaporated to dryness *in vacuo* and crystallized from aqueous pyridine and from pyridine/methanol/acetone/water, m. p. 262–264° decompn., ε 15,060. Final recrystallization from pyridine/acetone (2:3) diluted with water gave the standard sample, m. p. 262–264° decompn.; ε 15,224 ± 122; $[\alpha]_D^{23}$ + 67.1° (MeOH). The polarograph half-wave potential was −1.02 v. (*versus* saturated calomel electrode), the diffusion current, $i_D/C \pm \sigma$, at −1.10 v. being 4.39 ± 0.04 μa./mg./ml., at −1.25 v. being 4.54 ± 0.02 μa./mg./ml.

TRIAMCINOLONE 16α,21-DIACETATE

Identification.—Triamcinolone 16α,21-diacetate is characterized by melting point behavior, optical rotation, etc., in Table IV. Here again melting point behavior is of limited use in identification, although the wide melting range is characteristic of triamcinolone diacetate. At least two polymorphs have been prepared, as evidenced by infrared spectra in the solid state. The crystalline forms are interconvertible by using the appropriate solvent. Type I polymorph (from methylene chloride, chloroform, etc.) and type II polymorph (from acetone) are typified by the spectra of Fig. 2. Infrared absorption spectra of the diacetate in chloroform have recently been published (25).

TABLE IV.—PHYSICAL PROPERTIES OF TRIAMCINOLONE 16α,21-DIACETATE

Property	Bernstein, *et al.*, *J. Am. Chem. Soc* **78,** 5693., (1956).	Thoma, *et al.*, *J. Am. Chem. Soc.*, **79,** 4818 (1957).	This Report
Melting point (capillary)	158–235°[a]	179–180° with gas evolution	145–236°[b]
Specific Rotation	$[\alpha]_D^{25}$ + 22° (chloroform)	$[\alpha]_D^{23.5}$ + 28° (chloroform)	$[\alpha]_D^{27}$ + 22° (chloroform) + 63° (MeOH)
$\lambda_{max.}^{EtOH}$ (ε)	239 mμ (15,200)	....	239 mμ (15,270)
Infrared absorption (KBr disk)[c]	3,635, 3,440, 1,720, 1,674, and 1,630 cm.$^{-1}$[d]	....	2.90, 3.40, 5.74, 6.00, 6.15, 6.19, (shoulder) μ
$\lambda_{max.}^{0.095\ N\ NaOH\ in\ EtOH}$ (ε)	....	....	240 mμ (13,800), 265 mμ (7,150 inflection), 310 mu (2,000)[e]

[a] Bernstein, *et al.*, note occasional melting points of 186–188° with gas evolution and mention apparent solvation of the diacetate. Also m. p. 150–240° with loss of solvent is reported in U. S. pat. 2,789,118.

[b] The melting range for type II crystal; for type I, 185–232° decompn.

[c] Spectra in chloroform have been published; see (25).

[d] Proving the analytical sample of Bernstein, *et al.*, with the criteria of polymorphism described herein indicates a type I crystal.

[e] Spectra taken three to five minutes after solution at room temperature. At 60° for one hour, 240 mμ (14,500), 261 mμ (8,750, inflection), 310 mμ (3,280), not altered after two more hours at 60°.

Type I polymorph has a slightly different melting point behavior from type II, type I starting to melt later than type II (185–232° *versus* 145–235°). Type I polymorph has been obtained from chloroform, methylene chloride, acetone, acetone/petroleum ether, and benzene/petroleum ether. Type II has been obtained from acetone and from acetone/petroleum ether.

Identification of the diacetate may also be made using spectra in concentrated sulfuric acid (Table V). Characteristic spectra have also been obtained in 100% phosphoric acid (26). Bands at 260 mμ (E 1%, 1 cm. 299), 290 mμ (213, inflection), 310 mμ (205 inflection) and 375 mμ (144) characterize the diacetate in 100% phosphoric acid.

Determination.—Instrumental determination of triamcinolone diacetate may be made using the same methods that were described for the alcohol.

TABLE V.—SELECTIVE ABSORPTION SPECTRA OF TRIAMCINOLONE AND TRIAMCINOLONE DIACETATE IN CONCENTRATED SULFURIC ACID

	Time After Solution	$\lambda_{max.}$ ($E_{1\ cm.}^{1\%}$)	$\lambda_{min.}$ ($E_{1\ cm.}^{1\%}$)
Triamcinolone	15 min.	260 mμ (334)	220 mμ (63)
		310 mμ (172)	287 mμ (128)
		390 mμ (42)	345 mμ (16)
	2 hr.	260 mμ (346)	218 mμ (71)
		310 mμ (176)	288 mμ (142)
		380 mμ (86)	342 mμ (34)
	20 hr.	263 mμ (386)	220 mμ (80)
		310 mμ (176)	300 mμ (166)
		375 mμ (94)	340 mμ (40)
Triamcinolone 16α,21-diacetate	15 min.	261 mμ (297)	220 mμ (59)
		308 mμ (136)	283 mμ (88)
	2 hr.	261 mμ (297)	220 mμ (59)
		308 mμ (136)	283 mμ (88)
		380 mμ (35)	345 mμ (17)
	20 hr.	260 mμ (302)	217 mμ (82)
		308 mμ (137)	285 mμ (109)
		375 mμ (187)	335 mμ (50)
		475–480 mμ (10) Infl.	

Triamcinolone 16α,21-Diacetate Purification.—A selected sample of chromatographically purified triamincolone diacetate, m. p. 148–190° decompn., $\lambda_{max.}^{EtOH}$ 239 mμ (ε 15,400), homogeneous on papergrams, infrared spectra polymorph type I was recrystallized from acetone/petroleum ether (Skellysolve B), m. p. 145–200° decompn., ε 15,200, type II polymorph. Recrystallization from chloroform/petroleum ether yielded type I polymorph, m. p. 185–232° decompn., ε 15,000. Still further recrystallizations from acetone/petroleum ether and from acetone failed to alter the characteristic melting range. After drying *in vacuo* over refluxing toluene the sample melted 145–235° decompn., with ε 15,270, type II polymorph. The polarographic half-wave potential was −1.01 v., the diffusion current at −1.30 v. was 3.55 μa./mg./ml.

EXPERIMENTAL

Ultraviolet absorption spectra were obtained in absolute ethanol using the Cary Recording Spectrophotometer, Model 11S; molecular extinction coefficients were calculated from replicate measurements using the Beckman Spectrophotometer, Model DU. Optical rotations were determined on *ca.* 0.5% solutions in the appropriate solvent. Sulfuric acid spectra were determined according to published procedures (10, 27). Infrared spectra were obtained on solid samples as potassium bromide disks, using the Perkin-Elmer Model 21 double beam instrument.

Polarographic Solvent.—A solution of 0.1 *M* tetra-*n*-butylammonium hydroxide in 50% aqueous methanol is adjusted to pH 3.0 with phosphoric acid. The solution is stable indefinitely.

Polarographic Determination.—A 10-mg. sample of triamcinolone is weighed into a 10-ml. volumetric flask and 8 ml. of polarographic solvent is added. The mixture is shaken and warmed on a steam bath until solution is complete, cooled, and made to volume with polarographic solvent and mixed thoroughly. The solution is placed in the semimicro

polarographic cell and the capillary adjusted, etc. The current-voltage relationship is determined up to about −1.4 v. using the Leeds and Northrup Electrochemograph instrument at a suitable sensitivity setting. The diffusion current is determined from the reading at −1.10 v. (half-wave potential is −1.02 v.). The purity of the sample is obtained by comparison of the observed diffusion current with that of a standard run at the same time. The related Δ^4-3-ketosteroids exhibit a half-wave potential of *ca.* −1.20 v.

Tetrazolium Blue Reagent.—Five hundred milligrams of tetrazolium blue is dissolved in 100 ml. of absolute ethanol. The solution is filtered into an amber glass-stoppered bottle. Ten milliliters of a 10% solution of tetraethylammonium hydroxide is diluted with 100 ml. of absolute ethanol and filtered. The two solutions are stored in the refrigerator during the course of a series of determinations. In no case should the solutions be retained after two weeks, and fresh tetrazolium blue reagent made daily is recommended.

Colorimetric Determination.—Ten milligrams of triamcinolone is weighed into a 10-ml. volumetric flask, 8 ml. of absolute ethanol is added, and solution effected by vigorous shaking. Heat may not be applied to aid solution at this point as decreased absorption in color intensity invariably results, probably due to isomerization of triamcinolone. When solution is complete the sample is made to volume with ethanol and mixed. A suitable dilution is made to give about 40 μg./ml. of steroid. A 1-ml. aliquot of the 40 μg./ml. solution is placed in a 10-ml. volumetric flask, 1 ml. of tetrazolium blue reagent is added, followed by 1 ml. of the 1% tetraethylammonium hydroxide solution, and the solution is diluted to the mark with ethanol. After thirty minutes the absorbance is determined at 510–530 mμ (maximum *ca.* 525 mμ) using the Beckman DU instrument *versus* a reagent blank similarly treated, Results are calculated with reference to standard triamcinolone run at the same time. Using these techniques the standard deviation for a single determination was 0.0093, a series of 28 replicates gave $E(5\mu g./ml.)/1$ cm. (510 mμ) of 0.549 ± 0.009 for triamcinolone diacetate.

Determination of Triamcinolone in Tablets.—A sample of ten 2-mg. tablets of triamcinolone is pulverized in a mortar and triturated with a few milliliters of absolute ethanol. The sample is transferred to a 100-ml. volumetric flask, made up to about 80 ml. with absolute ethanol, and shaken on a rotary shaker for one hour. After making to volume with ethanol and mixing, the solution is centrifuged until a clear supernatant is had.

This solution is sampled for the several assays required. For polarographic analysis an aliquot containing 2–3 mg. of triamcinolone is transferred to a glass-stoppered flask and evaporated to dryness with gentle warming under a stream of air. The residue is dissolved in the calculated volume of polarographic solvent and the analysis made by polarographic means as already described. For colorimetric determination with tetrazolium blue, suitable dilutions are made of the clear ethanol solution and the colorimetric assay made directly without intermediate removal of the solvent. Ultraviolet absorption measurements may also be made on the clear ethanol solution, a suitable dilution to about 10 μg./ml. being made, and the absorbance at 239 mμ taken on the Beckman DU instrument.

Comparison of the three assays on 4-mg. tablets of triamcinolone gave polarographic 4.19 mg., spectrophotometric 4.16 mg., colorimetric (tetrazolium blue) 3.98 mg.

Porter-Silber Determination.—The directions of Porter and Silber (23) are followed, using ethanol solutions of the steroid at concentrations ranging from 1.7 to 12 μg./ml. Three milliliters of steroid solution is mixed with 24 ml. of the phenylhydrazine reagent (65 mg. of phenylhydrazine hydrochloride in 62 ml. of concentrated sulfuric acid and 38 ml. of water), heated at 60° for twenty minutes, cooled, and absorption spectra recorded over the range 380–500 mμ using a 5-cm. cell (*cf.* Table II).

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A Study of the Effect of Some Emulsifying Agents on Drug Release from Suppository Bases*

By CLYDE W. WHITWORTH and JOSEPH P. LAROCCA

Suppositories consisting of varying amounts of hydrogenated cottonseed oil, Tweens, Spans, and Arlacels were incorporated with a dye and tested for the percentage of dye release at time intervals. Most of the suppositories were tested for storage qualities and seven were subjected to *in vivo* tests. Several of the proposed bases appear to be very promising and compare favorably *in vivo* with a commercial brand of suppository now being widely used.

SUPPOSITORIES, one of the oldest forms of medication, have been the object of much experimentation. This is due to the fact that to be practical and useful suppository bases must give satisfactory drug releases, have good appearance and storage qualities, be neutral and nonirritating, and be compatible with all medication.

Until recent years theobroma oil and a few other fatty substances were accepted as the most satisfactory suppository bases. These fat-type bases had many disadvantages. Eiler (1) points out that oil soluble drugs when incorporated in fat-type bases (unless emulsified) tend to be released very slowly from the fat. Ohmart (2) states also that a fat-type base hinders the action of water-soluble medicaments by coating the mucosa of the body cavity. On the other hand, water-soluble drugs tend to be more readily released for either local or systemic effect when incorporated into a water-miscible base.

Theobroma oil and other like substances were used as suppository bases mainly because they melted at body temperature. This particular property is assuming less importance in face of the latest research in this field. It is quite apparent that the nature of the base is a determining factor in the rate of release of a drug for systemic medication. Gross and Becker (3) reported several new suppostitory bases in which emulsifying agents increased the rate of drug release. Del Pozo (4) found that Lanette Wax and Aerosol OT increased the rate of dialysis of potassium iodide from cocoa butter *in vitro*. In studying the influences of suppository bases upon the rectal absorption of acetylsalicylic acid, Cacchillo and Hassler (5) reported good absorption from Carbowax bases, fair absorption from theobroma oil bases, and poor absorption from glycerinated gelatin bases. An excellent review of suppositories, both old and new, is given by Gross and Becker (3).

In this study the line of endeavor was to produce acceptable suppository bases consisting of hydrogenated cottonseed oil and some of the newer synthetic emulsifying agents. Hartman and LaRocca (6) conducted a study on the use of mixtures of completely and partially hydrogenated cottonseed oil as suppository bases, obtaining good results. Coto Flakes,[1] which is completely hydrogenated cottonseed oil has a melting range of 58 to 62° and an iodine number of approximately 6. Cotmar,[2] which is partially hydrogenated cottonseed oil, has a melting point of about 34° and an iodine number of about 70. The emulsifying agents used were Spans, Tweens, and Arlacels.[3] Spans and Arlacels are essentially partial esters of the common fatty acids, lauric, palmitic, stearic, and oleic, and hexitol anhydrides derived from sorbitol. They are lipophilic and are generally insoluble in water, but soluble in organic solvents. Tweens are polyoxyethylene derivatives of the Spans (7). They are hydrophilic. Schwartz, *et al.* (8), conducted investigations using patch tests on human subjects and found these emulsifying agents to be nonirritating. Feeding tests have also proved them harmless (7).

EXPERIMENTAL

About seventy-five different suppository bases were prepared during the course of this study. From the results of *in vitro* study seven bases were chosen to be studied *in vivo*, in an attempt to correlate the two methods of determining drug release. All were made by fusion of the constituents with a minimum amount of heat. The melting points were determined using the capillary rise method for class two materials as described in the *United States Pharmacopeia* XV (9). Table I shows the formulas for the most satisfactory suppositories and their melting points.

All the suppositories were observed at room temperature for at least one week and the appearance noted. Several of those thought suitable were subjected to further tests to determine storage qualities, in which one suppository was wrapped in aluminum foil and placed, along with an unwrapped

* Received March 13, 1958, from the Research Laboratories of the University of Georgia, School of Pharmacy, Athens.

[1] Supplied by the Proctor and Gamble Co.
[2] Supplied by the Proctor and Gamble Co.
[3] Supplied by the Atlas Powder Co.

TABLE I.—PER CENT COMPOSITION AND MELTING RANGE OF FORMULAS

No. Formula	Tweens					Spans			Arlacels				Cotmar	Coto Flakes	Melting Range, °C.
	20	40	61	81	85	40	60	80	20	60	80	85			
1	20	..	20	..	..	..	..	..	..	..	..	..	60	..	34–36
2	..	20	20	..	..	..	..	..	..	..	..	..	60	..	45–47
3	..	..	20	..	..	20	..	..	..	..	..	..	60	..	36–38
4	..	..	10	..	..	..	..	..	..	..	..	..	80	10	45–47
5	..	..	..	10	..	..	..	..	..	..	..	..	80	10	46–48
6	..	..	..	..	10	..	..	..	..	..	..	..	80	10	46–48
7	..	..	20	..	..	..	..	..	..	..	..	..	60	20	50–52
8	..	..	..	20	..	..	..	..	..	..	..	..	60	20	50–52
9	..	..	..	..	20	..	..	..	..	..	..	..	60	20	51–53
10	..	..	..	..	..	..	20	..	..	..	..	..	60	20	50–52
11	..	15	20	..	..	..	..	..	..	..	..	..	60	5	42–44
12	..	..	20	..	..	..	..	15	..	..	..	..	60	5	44–46
13	..	..	20	..	..	20	..	..	..	..	..	..	57.5	2.5	39–40
14	..	..	..	..	..	30	..	..	..	..	..	..	50	20	47–49
15	..	..	20	..	..	..	..	..	15	..	..	..	60	5	41–43
16	..	..	20	..	..	..	..	..	..	15	..	..	60	5	40–42
17	..	..	20	..	..	..	..	..	..	..	15	..	60	5	40–42
18	..	..	20	..	..	..	..	..	..	..	..	15	60	5	40–42
19	..	..	..	..	..	..	..	..	..	..	..	..	90	10	45–47

one, in an oven at 30° for approximately two months. Not all the bases were subjected to the two months of oven testing, but all were tested for stability at room temperature. In general, the proposed bases which were tested for storage compared favorably with several commercial suppositories.

In Vitro Test for Drug Release and Storage Tests.—An exact amount of a dye, FD&C Red No. 2, representing a water-soluble medicinal agent was incorporated into one suppository. After twenty-four hours of refrigeration the suppository was ready for testing. The suppository containing the dye was placed in one percolator of water at 37° and a blank suppository in another. Low speed stirrers maintained circulation. At definite time intervals samples of water were withdrawn from each percolator and the dye release determined colorimetrically. The reading of the blank was corrected each time for the effect of the base alone on the transmittance of light. The actual percentage of dye released was determined from a graph previously prepared with known concentrations of the same dye. The procedure is almost identical to that used by Hartman and LaRocca (6) who give the method in detail. Table II shows the percentage of dye released for each formula tested.

In Vivo Testing of Suppositories.—A commercial brand of pentobarbital sodium suppository with a theobroma type base was tested and the time for onset of action recorded. Thereafter, four of the proposed bases which showed good dye release were incorporated with pentobarbital sodium and tested in a like manner. Two bases showing poor dye release were tested in the animals also. Female rabbits were used in the study and 10 animals were used in each determination. Loss of righting reflex was taken as the onset of action. Table III shows the results of the *in vivo* tests.

DISCUSSION

Many different formulas for suppository bases were studied. All contained hydrogenated cotton-

TABLE II.—DYE RELEASE

Formula No.	Per Cent Dye Release—Time in Minutes							
	2	4	6	8	10	12	14	30
1	42.5	42.5	43	43.5	44.3	44.3	44.3	44.3
2	25.7	38.5	38.5	38.5	38.5	39.3	39.3	39.3
3	2.9	2.9	20	32.8	37.6	39.4	39.4	41.8
4	2.8	4.7	11.8	17.9	23.9	26.4	30.7	42.1
5	1.0	4.3	7.1	11.1	12.5	14.3	15.5	21.4
6	2.1	3.9	7.9	12.5	16.1	21.4	23.5	38.5
7	1.0	2.1	2.1	2.1	3.6	4.3	4.3	5.7
8	0	2.0	3.4	5.7	7.0	7.5	8.0	10.0
9	2.8	2.8	4.3	5.0	5.0	5.7	6.0	12.5
10	0	0	0	0	0	0	1.4	2.0
11	5.1	5.1	7.5	14.6	17.5	28.0	33.2	55.4
12	1.4	3.9	4.3	16.0	25.0	41.0	42.8	71.4
13	0.7	0.7	31.4	45.7	45.7	45.7	45.7	45.7
14	0	0	0	0	0	0	0	1.0
15	2.8	5.7	24.6	35.7	40.7	46.5	53.5	57.8
16	0	0.7	2.1	2.1	5.0	10.0	28.5	47.8
17	1.4	3.2	7.1	11.4	18.5	22.0	25.0	38.5
18	2.1	3.5	5.7	14.2	17.1	20.7	22.2	52.2
19	0	0	0	0	0	0	0	0

TABLE III.—A COMPARISON OF THE RECTAL ABSORPTION FROM SOME PROPOSED BASES WITH A COMMERCIAL BRAND SUPPOSITORY IN RABBITS

Formula No.	No. of Rabbits Used	Average Time for Loss of Righting Reflex in Animals, min.	Standard Deviation, min.
3	10	11.5	±1.84
4	10	14.9	±3.82
10	10	Ineffective in 30 min.	...
11	10	8.3	±1.57
12	10	11.0	±1.89
14	10	Ineffective in 30 min.	...
15	10	9.9	±2.27
X[a]	12	16.4	±5.28

[a] A commercial brand of suppository with a theobroma oil base.

seed oil as the main constituent and varying amounts of surface active agents to increase the release of a drug as represented by a dye.

The percentages of completely and partially hydrogenated cottonseed oil were varied to control the melting range of the bases. The concentrations of emulsifying agents were varied to try to determine the amounts of these that would produce maximum dye release. The most desirable base would be one that contained a high percentage of hydrogenated cottonseed oil, which is relatively inexpensive, a high melting range for storage qualities, and a minimum amount of emulsifying agents that would produce acceptable drug release.

As was expected, the bases containing the Tween products gave the best dye release and also proved effective *in vivo*. This was probably due to the fact that the Tweens are hydrophilic and the dye and drug used were water soluble. The Span products gave somewhat less effective dye release. Some formulas containing both Spans and Tweens gave the best dye release. Four of the bases showed better than 50% dye release in thirty minutes. The *in vivo* tests compare seven proposed formulas with a commercial suppository as to onset of action.

When the *in vivo* and *in vitro* results are compared, the average times for loss of righting reflexes in the rabbits corresponds to a dye release of 31 to 40% in four of the five bases which were most effective *in vivo*. Two bases which gave poor dye release were ineffective *in vivo*.

The most effective base in the *in vivo* test gave a loss of righting reflex in approximately half the time of the commercial suppository. The action of the commercial suppository was somewhat erratic as shown by the standard deviation.

The results in this study cannot be compared precisely with other studies using the same method of *in vitro* tests because the composition of formulas are not the same. However, certain comparisons can be made. Gross (3) found that certain surface active agents when incorporated into theobroma oil increased dye release. Hartman and LaRocca (6) found almost no dye release from bases containing only hydrogenated cottonseed oil; these results were duplicated in this study. In general, other studies indicate that emulsifying and surface active agents increase drug release.

The results indicate that dye release is highest in bases that contain 35–40% of the emulsifying agents and have a melting range of less than 48°. Above this melting range the dye release and effectiveness *in vivo* drops off sharply.

All the suppositories reported here had a smooth, pleasing appearance and were quickly and easily made.

SUMMARY AND CONCLUSION

Nineteen suppository bases are given. Several of the bases show good release of a water-soluble active ingredient both *in vivo* and *in vitro*.

Judging from the dye release tests it seems that those bases containing 35–40 per cent of certain of the emulsifying agents gave best release. The Tween products seem ideally suited as ingredients in suppository bases and several of the bases appear to be superior to theobroma oil as a systemic suppository base.

Seemingly, more effort should be made to correlate *in vivo* and *in vitro* testing of suppositories.

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Antimicrobial Substances from Seeds*

By JASPER C. MARUZZELLA and MARTIN FREUNDLICH

One hundred and ninety-five extracts were prepared from 39 seeds of drug origin and screened *in vitro* against the following test organisms: *Escherichia coli, Staphylococcus aureus, Serratia marcescens, Mycobacterium smegmatis, Candida albicans, Erwinia caratovora,* and *Streptomyces Venezuelae.* The di-ethyl ether and acetone soluble fractions were found to possess greater antimicrobial activity than the ethyl alcohol, *n*-butyl alcohol, and water-soluble fractions. Sixteen of the 39 seeds were found to have no antimicrobial activity. Extracts of cardamom (green), tonka angostura, and celery were found to have antimicrobial activity on at least 6 of the 7 test organisms as well as against 12 additional microorganisms. Concentration of the extracts to less than one-half the original volume did not increase antimicrobial activity.

THE SCREENING of higher plants for antimicrobial substances has been an active area of investigation for the past two decades. Among the more complete of these studies are those of Osborn (1), Lucas and Lewis (2), Huddelson, *et al.* (3), Sanders, *et al.* (4), Carlson, *et al.* (5), and Okazaki, *et al.* (6–11). These investigators have tested more than 4,000 higher plants with about 20 per cent exhibiting some antibacterial activity. Other preliminary surveys are those of Atkinson and Rainsford (12), Hayes (13), Carlson, *et al.* (14), and more recently Masilungan, *et al.* (15), Atkinson (16), Ferenczy and Gracza (17), and Hasegawa, *et al.* (18). The parts of the plants examined for activity were: leaves, stems, roots, barks, flowers, entire plant, fruits, and occasionally seeds. Why seeds should be somewhat neglected in these surveys is not known. This does not mean that seeds have not been investigated, for indeed they have (19–25).

Similarly, screening for antimicrobial substances from plant drugs has been investigated (26–31) with little emphysis placed on seeds. This investigation therefore is concerned with extracting antimicrobial substances from seeds of drug origin.

MATERIALS AND METHODS

Test Organisms.—The bacteria employed were: *Escherichia coli, Staphylococcus aureus, Serratia marcescens, Mycobacterium smegmatis, Erwinia caratovora, Streptomyces Venezuelae, Sarcina lutea, Bacillus subtilis, Neisseria perflava, Salmonella cholerae suis, Salmonella typhi murium,* and *Proteus vulgaris.* The fungi used were: *Candida albicans, Trichophyton mentagrophytes, Microsporum canis, Helminthosporium sativum, Cryptococcus rhodobenhani, Penicillium digitatum,* and *Epidermophyton interdigitale.* All of the bacteria were cultivated in a nutrient broth medium consisting of: polypeptone 5 Gm., beef extract 3 Gm., and distilled water to make 1 L. For the nutrient solid medium 15 Gm. of agar was added to this formula. The fungi were cultivated on Sabouraud's dextrose broth and agar (Difco).

Preparation of Extracts.—The solvents used for extracting antimicrobial substances from the seeds were: distilled water, ether, acetone, alcohol, and *n*-butyl alcohol. The extraction procedure was conducted as follows: Twenty grams of dry seeds was added to 50 cc. of solvent and allowed to soak for one hour. The material was placed into a Waring Blendor and macerated for two minutes, then filtered through filter paper.

Method of Testing.—The filter paper disk method of Vincent and Vincent (32) was used to determine the presence of antimicrobial activity. In this method 1 cc. of a twenty-four hour broth culture of bacteria was placed into Petri dishes with approximately 10 cc. of nutrient agar. In the case of the fungi, 1.5 cc. of a two to three-day old culture were placed into Petri dishes together with about 10 cc. of Sabouraud's dextrose agar. Filter paper disks (6.35 mm. diameter) were saturated with the extract and allowed to air dry. This procedure was repeated so that each disk contained four applications of the extract. The disks thus prepared were placed on the surface of the seeded agar. All dishes with bacteria were incubated at 37° for twenty-four hours except *E. caratovora* (forty-eight hours at room temperature) and *S. Venezuelae* (twenty-four hours at room temperature). All of the dishes with fungi were incubated at 37° for three days except *H. sativum* which was incubated for two days at room temperature. Zones of inhibition surrounding the disks were measured with a mm. ruler and an illuminated Quebec colony counter. Six disks were prepared for each extract and tested against each organism. Zones of inhibition recorded represent a mean value of six readings.

Additional extracts were prepared in the same manner as described above but the final volume of these extracts was concentrated (by evaporation) to less than one-half its original volume. These concentrated extracts were also tested for antimicrobial activity.

RESULTS AND DISCUSSION

One hundred and ninety-five extracts were prepared from 39 seeds and screened against seven test

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All of the seeds used in this investigation were generously supplied by S. B. Penick Company, New York City.

TABLE I.—ANTIMICROBIAL ACTIVITY OF SEED EXTRACTS

Seeds	Zone of Inhibition in mm.[a]						
	E. coli	*S. aureus*	*S. marcescens*	*M. smegmatis*	*C. albicans*	*E. carotovora*	*S. Venezuelae*
Ambrette[b] (wa)	0[c]	0	0	2	0	0	0
Angelica (ac)	0	2	0	2	3	2	5.5
Angelica (ba)	0	2	0	2	3	2	5.5
Anise (ac)	0.5	2	1.5	3	4	1	4
Annatto (ee)	0	1	0	0	0	0	0
Annatto (ac)	0	1	0	0	0	0	0
Annatto (ea)	0	1	0	0	0	0	0
Annatto (ba)	0	1	0	0	0	0	0
Asparagus (wa)	2	2	0	1.5	0	1	2
Caraway (ea)	0.5	0.5	0.5	0	2	0.5	4
Caraway (ba)	0.5	0.5	3	0	2	0.5	2
Cardamom, green (ee)	4	2	0.5	5	4	0.5	6
Cardamom, green (ac)	4	3.5	0.5	5.5	3	0.5	4.5
Cedron (ea)	0.5	0	0	2.5	0	1	0
Celery (ee)	0	2	1	1	5	3.5	8
Coriander (ee)	2	0	0	2	0	1	4.5
Coriander (ac)	2.5	0	0	2	0	1	4.5
Cumin (ee)	3	0	0.5	3	3	0	5
Dill (ea)	0	0	0	0	0.5	0.5	4.5
Dill (ba)	0	0.5	0	0	0.5	0	4
Fennel (ee)	2.5	1.5	1.5	1.5	4	6	5
Fennel (ac)	2	1.5	2	1.5	4	6	5
Fennel (ea)	1	2	2	1.5	4.5	6	5
Fennel (ba)	2.5	1	1.5	1.5	4.5	6	5
Jambul (wa)	0	3	0	1.5	0	3	4
Larkspur (ee)	0	1	0	0.5	1.5	1.5	1
Mustard, yellow (wa)	0	0	0	0	0	0	2
Nigella (ac)	1	8	1	1	2	1.5	9
Nux Vomica (wa)	0	0	0	0	0	0	0.5
Nux Vomica (ea)	0	0	0	0	0	0.5	0
Nux Vomica (ba)	0	0	0	0	0	0.5	0
Sabadilla (ea)	1	0	0	0	2	0	2
Sabadilla (ba)	1	0	0	0	2	0	1.5
Stavesacre (wa)	0	0	0	0	0	0	5
Sunflower (wa)	0	0	0	2	0	0	0
Tonka angostura (ee)	2	0	2.5	1.5	5	3	8
Wormwood American (ee)	4	1.5	0	1	2	1	4
Wormwood American (ea)	4	2	0	1	4	1	4

[a] Measurement from disk edge to zone edge.
[b] wa—water extract, ee—ether extract, ac—acetone extract, ea—alcohol extract, ba—*n*-butyl alcohol extract.
[c] Zone of inhibition absent.

organisms. The extracts from 23 seeds which were found to possess greatest antimicrobial activity are listed in Table I together with the solvents used for extraction and zones of inhibition. Extracts prepared from the following seeds were found to be inactive against the seven test organisms: calabar bean, conium, fenugreek, flax, hemp, millet, mustard (black), psyllium, pumpkin (select), quince, rape, sesame, stramonium, strophanthus, watermellon, and thistle (black). The ether and acetone soluble fractions possessed the greatest antimicrobial activity. It was found that *E. coli* was most susceptible to ether, acetone, and alcohol extracts of cardamom (green) and wormwood (American); *S. aureus* to ether, acetone, alcohol, and *n*-butyl alcohol extracts of nigella; *S. marcescens* to ether, acetone, alcohol, and *n*-butyl alcohol extracts of tonka angostura; *M. smegmatis* to ether and acetone extracts of cardamom (green); *C. albicans* to ether and acetone extracts of celery and tonka angostura; *E. carotovora* to ether, acetone, alcohol, and *n*-butyl alcohol extracts of fennel; and *S. Venezuelae* to ether, acetone, alcohol, and *n*-butyl alcohol extracts of nigella, celery, and tonka angostura.

It is interesting to note that the essential oils obtained from the seeds of anise, caraway, cardamom, celery, coriander, dill, fennel, and wormwood have been reported to possess antimicrobial properties with the filter paper disk method (33, 34). In this investigation extracts of these seeds were also found to possess antimicrobial properties. Whether the essential oil contained in these seeds is responsible for microbial inhibition has not been determined in this study. However, Ferenczy (22) has presented evidence to indicate that the antibacterial activity of petroselinum seeds is due to its volatile oil content.

Nine extracts of different seeds showing marked antimicrobial activity against the organisms screened in Table I were selected and concentrated. Upon screening these concentrated extracts on the seven test organisms it was found that the zones produced did not differ appreciably from the unconcentrated extracts. These concentrated extracts were further tested against 12 additional microorganisms, the results of which are found in Table II. From these data it is seen that the acetone-soluble fraction of cardamom (green) as well as the ether-soluble fractions of tonka angostura and celery possess a very wide spectrum. The activity of tonka angostura may be attributed to its coumarin content. For indeed coumarin has been reported to possess antimicrobial properties (35, 36).

TABLE II.—ANTIMICROBIAL ACTIVITY OF CONCENTRATED SEED EXTRACTS

	Zone of Inhibition in mm.[a]											
Seeds	*S. lutea*	*B. subtilis*	*N. perflava*	*S. cholerae suis*	*S. typhi murium*	*P. vulgaris*	*T. mentagrophytes*	*M. canis*	*H. sativum*	*C. rhodobenhani*	*P. digitatum*	*E. interdigitale*
Anise (ac)[b]	4	2.5	0[c]	0.5	0.5	1.5	1.5	2	4.5	1	1.5	1
Cardamon, green (ac)	8	5	3	5	4.5	8	5	3.5	8	4	5	5
Celery (ee)	7	5.5	1	1	1	5	4	6	7	3	3.5	4
Coriander (ee)	2	0.5	1	0	1	5	0	0.5	3.5	1	0.5	1
Fennel (ac)	10	1.5	0	0	0	3	14	1	5.5	5	5	10
Nigella (ac)	20	2	0	0	0	2	9	5	5	3	8	10.5
Sabadilla (ea)	9	1	0	0	0	0	2	0	0	0	2	5
Tonka angostura (ee)	8	2	1.5	2.5	1.5	1	11	1	8	9	9	13
Wormwood, American (ee)	6	1	1	3	0	2	13	2	6	7	6	14

[a] Measurement from disk edge to zone edge.
[b] ac—acetone extract, ee—ether extract, ea—alcohol extract.
[c] Zone of inhibition absent.

SUMMARY

1. One hundred and ninety-five extracts were prepared from 39 seeds of drug origin and screened against the following test organisms: *Escherichia coli, Staphylococcus aureus, Serratia marcescens, Mycobacterium smegmatis, Candida albicans, Erwinia caratovora,* and *Streptomyces Venezuelae.*

2. The solvents used for extraction were ether, acetone, alcohol, *n*-butyl alcohol, and water. The ether and acetone-soluble fractions were found to have the greatest antimicrobial activity.

3. Sixteen of the seeds were found to possess no antimicrobial activity with all extracts prepared while the remaining 23 seeds were found to be active.

4. Extracts of cardamom(green), tonka angostura, and celery were found to have antimicrobial activity on at least six of the seven test organisms as well as against all of the following: *Sarcina lutea, Bacillus subtilis, Neisseria perflava, Salmonella cholerae suis, Salmonella typhi murium, Proteus vulgaris, Trichophyton mentagrophytes, Microsporum canis, Helminthosporium sativum, Cryptococcus rhodobenhani, Penicillium digitatum,* and *Epidermophyton interdigitale.*

5. Antimicrobial activity was not increased by concentrating the extracts.

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Analysis of Barbiturate Salts in Various Dosage Forms by an Ion Exchange and Nonaqueous Titration Procedure*

By MURIEL C. VINCENT and MARTIN I. BLAKE

Barbiturate salts in various dosage forms are determined by liberating the barbituric acid from the salt by passage through a cation exchange resin. Dimethylformamide is used as the solvent. The barbituric acid is titrated visually with 0.1 *N* sodium methoxide in benzene-methanol. The method is rapid and avoids the tedious extraction procedure.

SUCCESSFUL TITRATION of barbiturates in nonaqueous solvents has been reported by numerous workers. Vespe and Fritz (1) determined barbiturates, pure and in dosage forms, by visual titration in dimethylformamide using sodium methoxide in benzene-methanol. It was noted that for tablets, the barbiturate may either be extracted prior to titration, or the powdered tablets may be titrated directly.

Pifer, Wollish, and Schmall (2) describe a procedure for the nonaqueous titration of barbiturates. Sodium salts of the barbiturates are determined by liberating the barbituric acid with sulfuric acid, followed by extraction with an organic solvent. Dimethylformamide is added and the solution is titrated with sodium methoxide.

Swartz and Foss (3) titrated barbiturates, as free acids, in a polyethylene glycol 400 and chloroform mixture. Titration was effected potentiometrically with sodium methoxide. Salts were extracted with chloroform, after acidification, using a Schmall extractor. The extracted barbituric acid was titrated by the proposed method.

Ryan, Yanowski, and Pifer (4) used lithium methoxide in benzene-methanol as titrant for barbituric acids and their salts. Salts, capsules, and tablets were extracted, after acidification, in a Schmall extractor or Squibb separator. The extracted barbiturates were titrated with lithium methoxide.

Chatten (5) titrated barbituric acids visually with potassium hydroxide in methanol, using chloroform as the solvent. It is indicated that barbiturate salts may be analyzed by liberation and extraction of the barbituric acid.

Ion exchange resins have been applied to the analysis of barbituric acids (6, 7), but not to the salts.

This report describes a procedure for the analysis of barbiturate salts, pure and in various dosage forms. A solution of the salt in dimethylformamide is passed through a cation exchange resin. The effluent, containing the barbituric acid, is titrated visually with sodium methoxide in benzene-methanol. The indicator is azo violet.

EXPERIMENTAL

Preparation of Column.—The cation exchange resin Amberlite IRC-50 was used in this study The column was prepared as reported in a previous paper (8).

Nonaqueous Titration.—All titrations were effected visually with 0.1 *N* sodium methoxide in benzene-methanol, prepared and standardized as described earlier (9). The indicator was azo violet (saturated solution of *p*-nitrobenzene-azoresorcinol in benzene).

Assay Procedure.—*Pure Barbiturate Salts.*—Approximately 100 mg. of the barbiturate salt, accurately weighed, was dissolved in 20–25 ml. of dimethylformamide, and the solution was passed through the column of resin. Additional dimethylformamide was added to the column until 50 ml. of effluent was collected. The effluent was titrated visually to the first permanent blue color. With each series of five determinations a blank was run.

With each barbiturate salt several potentiometric titrations were conducted using a Fisher titrimeter equipped with a calomel and glass electrode system. Indicator solution was added and the color change was noted at the potentiometric end point. In each case, the color change which most nearly approximated the true end point, was the first permanent blue color.

Ampuls.—Samples containing about 100 mg. of barbiturate salt were used for analysis. Ampuls containing liquids were assayed by removing a suitable aliquot and diluting to about 25 ml. with dimethylformamide. The solution was passed through the column. Fifty milliliters of effluent was collected and titrated as described for barbiturate salts. Ampuls containing solid material were analyzed by dissolving the powder in a suitable volume of dimethylformamide. An accurately measured aliquot, containing about 100 mg. of barbiturate salt, was treated in the described manner.

Capsules and Tablets.—The contents of a suitable number of capsules or tablets were dissolved in dimethylformamide. The solution was filtered into a volumetric flask, the filter was rinsed with dimethylformamide, and the flask was made up to volume. An aliquot equivalent to about 100 mg. of barbiturate salt was removed and treated in the usual manner.

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The authors gratefully acknowledge the following companies for supplying samples of the barbiturates: Abbott Laboratories, Ames Company, Inc., Eli Lilly and Co., Merck Sharp and Dohme, McNeil Laboratories, Inc., Parke, Davis and Co., The Upjohn Co., and Winthrop Laboratories.

Suppositories.—A suitable number of suppositories was dissolved in dimethylformamide with the aid of gentle heat. The solution was cooled and filtered into a volumetric flask. The filter was washed with additional dimethylformamide and the flask was made up to volume. An aliquot containing about 100 mg. of barbiturate salt was analyzed as described above.

The results obtained for the recovery of barbiturate salts from various dosage forms are listed in Table I.

DISCUSSION

The successful determination of barbiturates by direct nonaqueous titration has been reported by numerous workers. It has been clearly demonstrated that the procedure is more rapid and less tedious than the official method, and is equivalent in accuracy and precision. The salts of the barbiturates, on the other hand, require an extraction procedure after conversion of the salt to the free acid, which is subsequently determined by nonaqueous titration in the usual manner. The analysis of barbiturate salts may be determined in glacial acetic acid by titration with perchloric acid, but Ryan, *et al.* (4), have indicated that the procedure is not feasible for other dosage forms because of interference due to the presence of diluents and excipients. The results of the present study indicate that a cation exchange resin can be used successfully for extraction of the free barbituric acid from the salt form by exchanging a hydrogen for the metal of the salt. The effluent is titrated with 0.1 *N* sodium methoxide. The method is applicable to a wide variety of dosage forms. With the exception of enteric coated tablets of pentobarbital calcium, good results were obtained as shown in Table I. No explanation can be offered for the poor precision reported for pentobarbital calcium. Application to the analysis of elixirs proved unsuccessful. A number of elixirs were studied, but consistent results were not obtained. Interfering ingredients and difficulty in removing the water from the elixir base probably contributed to the failure of the method. However, elixirs may be analyzed by employing the extraction procedure as in the official method.

Several advantages of the ion exchange modification are apparent. The method is more rapid and eliminates the tedious process of extraction. Once the column has been prepared at least 15 determinations can be effected before regeneration becomes necessary.

In the course of this investigation it was observed that blank corrections were very low, negligible in most cases. It appears that passing dimethylformamide through the resin column serves as a means of purifying the solvent. It is free of odor and acidic impurities.

TABLE I.—ANALYSIS OF BARBITURATES IN DOSAGE FORMS

Dosage Form	Recovery, %
Pure Salts	
Amobarbital sodium	99.32 ± 0.40[a] (3)[b]
Barbital sodium	100.19 ± 0.94 (4)
Butabarbital sodium	98.88 ± 0.37 (3)
Butallylonal sodium	100.92 ± 0.07 (2)
Cyclobarbital calcium	99.81 ± 0.72 (3)
Pentobarbital sodium	100.16 ± 0.21 (3)
Phenobarbital sodium	99.65 ± 0.36 (3)
Secobarbital sodium	100.71 ± 0.02 (2)
Thiopental sodium	100.22 ± 1.05 (4)
Ampuls	
Amobarbital sodium, 65 mg.[c]	100.36 ± 0.80 (3)
Hexobarbital sodium, 1 Gm.	99.47 ± 0.72 (3)
Pentobarbital sodium, 2.5 Gm.	100.11 ± 0.93 (3)
Phenobarbital sodium, 130 mg.	101.46 ± 0.77 (3)
Secobarbital sodium, 250 mg.	100.02 ± 0.61 (3)
Thiamylal sodium, 10 Gm.	99.76 ± 0.34 (3)
Vinbarbital sodium, 300 mg.	100.97 ± 0.22 (3)
Capsules	
Amobarbital sodium, 1 gr.	100.83 ± 0.83 (2)
Pentobarbital sodium, 100 mg.	99.79 ± 0.33 (3)
Secobarbital sodium, 1/2 gr.	100.73 ± 0.38 (3)
Suppositories	
Pentobarbital sodium, 200 mg.	99.36 ± 0.65 (4)
Secobarbital sodium, 1/2 gr.	100.85 ± 0.40 (3)
Tablets	
Amobarbital sodium, E.C., 200 mg.	99.75 ± 0.34 (3)
Pentobarbital calcium, E.C., 100 mg.	101.46 ± 2.79 (4)
Secobarbital sodium, E.C., 3/4 gr.	98.74 ± 0.47 (3)

[a] Based on labeled amount. [b] Number of determinations. [c] Labeled amount in unit dosage form.

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A Note on the Effects of Gibberellins on Alkaloidal Content of *Hyoscyamus niger**

By JOHN Y. MASUDA and GLENN H. HAMOR

A comparative assay was made for total alkaloids of the gibberellin-treated and untreated *Hyoscyamus niger* by the U. S. P. assay procedure. It has been shown that gibberellins do not increase the alkaloidal content in the leaves. The total alkaloidal content of the overground portion of the plant was somewhat less than that of the untreated plants.

THE PLANT-GROWTH stimulating properties of gibberellins, obtained from the fungus, *Fusarium moniliforme*, had been observed in Japan as early as 1926. It was not until recent years that exhaustive experimentations have been carried on in the United States.

The major effects of gibberellins on plants have been reported as producing increased rate of growth and increased size and weight (1). It has also been shown that gibberellins may replace light in plants grown under low light intensity (2).

Smith and Sciuchetti (3) reported decrease in total alkaloids in the leaves, tops, and stems of *Datura stramonium* which were treated with gibberellic acid in concentrations of 100 and 1,000 p. p. m. The roots, however, were observed to have increased alkaloid production. They also observed decrease in total alkaloids in the leaves, tops, stems, and roots of *Atropa belladona* which were sprayed with gibberellic acid three successive times at ten-day intervals. In this investigation a comparative assay was conducted on treated and untreated *Hyoscyamus niger* for total alkaloidal content. The plants used for this investigation were grown and obtained through the courtesy of Anton Lang of the Department of Botany, University of California, Los Angeles, and their identity certified by him.

Plants of *Hyoscyamus niger*, of a biennial, purple-flowered strain, were grown from seeds in a greenhouse at a temperature of 20° in which the photoperiod was kept at eighteen hours by supplementing natural light with artificial illumination. Crystalline gibberellin[1] was dissolved in distilled water to which a small amount of Tween 20 was added and an amount equivalent to 5 μg. was applied daily with a hypodermic syringe at the base of the leaf nearest the apex of the stem (4).

The leaves showed some discoloration and seemed to be weaker in appearance than the leaves of the untreated control plant. The most noticeable effect of of the gibberellin-treated plant was stem elongation, with resultant increase in the internodes. There was considerable increase in total dry weight in the treated plants due to increase in stem weight, but the weight of the leaves was shown to decrease slightly. The data on weight, height, and alkaloidal content are given in Table I. The figures shown are averages of 24 plants and the weights given are average weight per plant.

TABLE I

Age, Days	Dry Weight, Gm.	Alkaloids, % Sample 1	Sample 2	Sample 3	Height, Inches
		Treated Plants			
21	2.3				14.5
	Leaves, 1.12	0.171	0.166	0.168	
	Stem, 1.18	0.023	0.023	0.022	
28	2.55				21.5
	Leaves, 1.14	0.128	0.130	0.129	
	Stem, 1.41	0.014	0.016	0.017	
35	2.85				26.0
	Leaves, 1.13	0.127	0.126	0.129	
	Stem, 1.72	0.014	0.013	0.013	
42	3.14				36.5
	Leaves, 1.09	0.123	0.124	0.122	
	Stem, 2.05	0.005	0.006	0.006	
		Untreated Plants			
	Leaves				
21	1.23	0.169	0.178	0.171	6.0
28	1.24	0.142	0.146	0.140	6.1
35	1.26	0.144	0.142	0.139	6.2
42	1.29	0.153	0.150	0.158	6.5

The U. S. P. assay procedure was used but, because of the limited amount of sample, the weight of the samples used was one-half the required weight. The plants were harvested at intervals of seven days and were immediately dried for two days in a circulating oven at a temperature of 40°. This material was ground and analysis performed immediately.

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(1) Haber, A. H., and Talbert, N. E., *Plant Physiol.*, **32**, 152(1957).
(2) Leben, C., and Barton, L. V., *Science*, **125**, 494(1957).
(3) Smith, G. M., and Sciuchetti, L. A., THIS JOURNAL, **48**, 63(1959).
(4) Sachs, R. M., and Lang, A., *Science*, **125**, 1144(1957).

* Received June 21, 1958, from the School of Pharmacy, University of Southern California, Los Angeles.

[1] Supplied by Eli Lilly and Co., Indianapolis, Ind., containing gibberellin A_3 and up to 10% of unknown but apparently inactive materials.

Communication to the Editor

The Amount of Fines Necessary for a Tablet Granulation

Dear Sir:

Numerous articles, books, and advertising pieces discussing compressed tablet manufacture mention the importance of "fines." Occasionally, 10–20% is set as the maximum amount of "fines" permissible. It is interesting to note that "fines" are rarely defined.

We are of the opinion that there is no general limit to the amount of "fines" (whatever they may be) that can be present in a granulation. Objections to "fines" must be determined for each specific formula.

To illustrate that "fines," in the freely used sense, are not always objectionable, the following examples are listed.

A. Tablets of 3-phenylpropylcarbamate, 400 mg. each, were prepared. Sieve analysis of the granulation indicated that all material passed through a No. 30 screen and 50% through a No. 200 screen. The tablet was successfully compressed at approximately 300 tablets per minute on the Stokes Model B-2 rotary tablet machine.

B. A standard APC tablet using 40 mesh aspirin crystals and spray dried acetophenetidin (1) was prepared by direct compression. Sieve analysis indicated that all the material passed through a No. 30 screen, 84% through a No. 60 screen, and 62% passed through a No. 100 screen. The tablets were successfully compressed at approximately 1,400 tablets per minute on a Stokes Model BB-2 rotary tablet machine.

C. A placebo spray-dried calcium sulfate granulation was prepared. Sieve analysis indicated that 92% passed through a No. 100 screen. The tablet was successfully compressed at approximately 700 tablets per minute on a Stokes Model B-2 rotary tablet machine.

D. A standard wet-granulated calcium sulfate granulation was prepared. Sieve analysis indicated that all the material passed through a No. 30 screen and 84% through a No. 100 screen. The tablets were successfully compressed at approximately 2,000 tablets per minute on a Stokes Model BB-2 rotary tablet machine.

E. Tablets of chlorpromazine, 25 mg. each, were prepared by a standard wet granulating technique. Sieve analysis indicated that 20% of the material was retained on a No. 20 mesh screen, 40% retained on a No. 100 mesh screen, and 40% passed through a No. 100 mesh screen. The tablets were successfully compressed at approximately 1,800 tablets per minute on a Stokes Model BB-2 rotary tablet machine.

We do not wish to imply that "fines" are not an integral part of a granulation, nor do we wish to imply that they do not influence compression. For each specific granulation one must determine what portion (of the smaller mesh size) is unacceptable, and why it is unacceptable; this then could be termed excessive "fines."

(1) U. S. pat. 2,798,838, July 9, 1957.

Smith Kline and French Laboratories
Philadelphia, Pa.

STANLEY J. TUCKER
HENRY M. HAYS

Received April 14, 1959.

Book Notices

The Chemistry of Drugs. 3rd ed. By NORMAN EVERS and DENNIS CALDWELL. Interscience Publishers, Inc., New York, 1959. 415 pp. 15 x 23 cm. Price $12.25.

This is an entirely new book, the third edition published in 1933 having been completely revised. The new third edition deals with the chemistry of both synthetic and natural drugs, their structure, methods of preparation, and synthesis, and in addition, their chemical properties and therapeutic uses. Comprehensive tables of official or approved names of drugs together with their proprietary names and chemical names are also included. Approximately half of the book is devoted to synthetic drugs, and the other half to naturally occurring medicinal chemicals.

Textbook of Physiology and Biochemistry. By GEORGE H. BELL, J. NORMAN DAVIDSON, and HAROLD SCARBOROUGH. The Williams and Wilkins Co., Baltimore, 1959. xi + 1,065 pp. 16.5 x 24 cm. Price $12.50.

This is the fourth edition of a book that is intended to be an introduction to the study of physiology and biochemistry. In order to bring the text up to date, considerable rewriting has been done, even though the third edition was published only two years ago. The book is intended primarily for medical students, but should be useful to students in related fields. Numerous cross references correlate the information presented under different sections of the subject. References are given after each of the 55 chapters, and an index is appended.

Recent Studies in Yeast and Their Significance in Industry. S. C. I. Monograph No. 3. The Macmillan Co., New York, 1959. 162 pp. 14 x 21.5 cm. Price $2.95.

This book includes papers and discussions presented during a Symposium organized by the Dublin and District Section of the Microbiology Group and the Food Group held in Dublin in September 1956. Six sessions were held during the Symposium, during which recent advances in research on yeasts were presented.

Mono- and Sesquiterpenoids. Vol. II of *The Chemistry of Natural Products.* By P. DE MAYO. Interscience Publishers, Inc., New York, 1959. vii + 320 pp. 15 x 23 cm. Price $7.50.

The Higher Terpenoids. Vol. III of *The Chemistry of Natural Products.* By P. DE MAYO. Interscience Publishers, New York, 1959. vii + 239 pp. 15 x 23 cm. Price $6.

The first volume of this important series on the chemistry of the alkaloids was described briefly in THIS JOURNAL, **47,** 232(1958). Volumes II and III, dealing with the chemistry of the mono- and sesquiterpenoids of the higher terpenoids, respectively, have recently been published simultaneously. Both volumes represent an important contribution to the chemistry of these important and interesting compounds. The terpenoids provide a clear concept of the versatility of carbonium ion rearrangements and furnish a useful framework upon which many general chemical reactions may be studied. These include synthetic and degradative processes and furnish examples of the sterochemical requirements of reactions and of the relationship between sterochemistry and stability and other physical properties.

Methoden der Organischen Chemie (Houben-Weyl). Stickstoff-Verbindungen II/III. 4th ed. Band XI/2 Edited by EUGEN MÜLLER. Georg Thieme Verlag, Stuttgart, 1959. xlviii + 840 pp. 17 x 25.5 cm. Price DM 155, subscription DM 139.50.

This volume represents the tenth in a series of a completely new and revised fourth edition of "Methoden der Organischen Chemie (Houben-Weyl)," the first of which was published in 1952. The publication of the volumes have not run consecutively, and it is expected that at least fourteen will be required to complete the new revision. The first in the series was Band VIII, covering the third part of oxygen-containing compounds. The present volume, XI/2 contains a conclusion of the second part of XI/1 which was devoted to Nitrogen Compounds II. Band XI/1 was described in THIS JOURNAL, **47,** 612(1958). About one-third of Band XI/2 covers the transformation reactions of primary and secondary amines. Conversion reactions by which amines are transformed into other substances such as acid amides, amidines, nitramines, and hydroxamines are comprehensively presented, and deamination reactions are discussed. In addition, special acylation methods are competently discussed for the introduction of acid radicals such as formyl, acetyl, and benzoyl into amines.

Nitrogen Compounds III is composed of five chapters. In the first chapter, which is relatively short, the preparation and transformations of 1,2- and 1,3-alkylenimines are discussed. The second, and one of the largest chapters is devoted to methods for the preparation and transformations of amino acids and their derivatives. This chapter is particularly outstanding because of its completeness of treatment of the important group including amino acids. Analytical methods for these compounds are adequately covered.

The fourth chapter, which also is quite extensive, covers preparative methods and other pertinent information relating to quaternary ammonium compounds. The fifth and last chapter deals with nitrogen-sulfur compounds and follows the usual arrangement of the other chapters by providing a general introduction to the subject and information concerning methods of synthesis and transformation reactions. Volume XI/2 contains an author and a subject index, both of which are well designed and which enable the reader to locate information quickly.

Colorimetric Determination of Traces of Metals. 3rd ed. By E. B. SANDELL. Interscience Publishers, Inc., New York, 1959. xxii + 1,032 pp. 15 x 23 cm. Price $24.

The general plan of treatment of the subject in this third edition is the same as in the second edition, which was reviewed in This JOURNAL, **40,** 177(1951). Methods that have been published since 1950 were reviewed. Some have replaced former methods, and other older methods have been revised. The many references and the indexes enhance the value of this book to the analytical chemist.

Modern Pharmacognosy. By EGIL RAMSTAD. Blakiston Division, McGraw-Hill Book Co., Inc., New York, 1959. viii + 480 pp. 15 x 23 cm. Price $10.50.

This textbook presents drugs of biological origin with emphasis on their biochemical relationships. The drugs are arranged in chapters according to chemical classifications. The latter part of the book takes up commercial drug production, formation of drug constituents, variability in drug activity, preservation and storage, and analysis of natural drug products. An index is appended.

Cell and Tissue Culture. By JOHN PAUL. E. & S. Livingstone Ltd., Edinburgh and London, 1959. Distributed in the U. S. by the Williams & Wilkins Co., Baltimore. viii + 261 pp. 14 x 21.5 cm. Price $7.

An up-to-date account of the techniques and applications of the important subject of tissue culture is presented. The text is based on the instruction given at the Tissue Culture Association Summer Course, and has been written particularly with the needs of participants of that course in mind. The book is divided into four parts. In Part I, the principles of cell culture are outlined in a series of six chapters. This is followed by comparable treatment of subjects pertaining to the preparation of materials, special techniques, and applications of cell and tissue culture methods.

American Drug Index 1959. By CHARLES O. WILSON and TONY EVERETT JONES. J. B. Lippincott Co., Philadelphia, 1959. 671 pp. 13.5 x 20.5 cm. Price $5.75.

This is the fourth annual edition of a useful compilation of generic, trade, and chemical names and composition of drugs and dosage forms with indicated uses. Although the text has been brought as up to date as possible with the addition of new drugs, the elimination of less useful material has enabled the reduction from 716 pages (1958) to 671 pages.

New and Nonofficial Drugs 1959. Evaluated by the A. M. A. Council on Drugs. J. B. Lippincott Co., Philadelphia, 1959. xxvii + 687 pp. 12 x 19 cm. Price $3.35.

The 1959 edition of this annual publication of the Council on Drugs of the American Medical Association contains descriptions of drugs evaluated on the basis of available data and reports of investigations. Monographs presented under nonproprietary names are designed to provide: chemical or biologic identity, including pertinent properties; actions and uses, including associated side effects, toxicity, and precautions; dosage, including routes of administration; available preparations; and applicable commercial names. The value of this book to the medical and related professions makes each volume a necessary addition to all libraries.

The Effect of Pharmacologic Agents on the Nervous System. Vol. XXXVII. Edited by FRANCIS J. BRACELAND. The Williams & Wilkins Co., Baltimore, 1959. xi + 488 pp. 15.5 x 23 cm. Price $13.50.

The Association for Research in Nervous and Mental Disease held a symposium in 1957 on "The Effect of Pharmacologic Agents on the Nervous System." This book constitutes the proceedings of the symposium and contains reports by outstanding authorities on clinical experience, evaluations, and aspects of research activity. It also includes reviews of the literature.

Antibiotics Annual 1958–1959. Edited by HENRY WELCH and FELIX MARTI-IBAÑEZ. Medical Encyclopedia, Inc., New York, 1959. xvii + 1,107 pp. 17 x 25 cm. Price $12.

This annual publication reports the Proceedings of the Sixth Annual Symposium on Antibiotics which was held in Washington, D. C., October 15, 16, and 17, 1958. These dates coincided with the thirtieth anniversary of the discovery of penicillin in London's St. Mary's Hospital. It was, therefore, appropriate that the first session of the Symposium was devoted to a series of papers on the history and development of antibiotics. During other sessions, a large number of papers on recent advances in the field of antibiotics was presented. Altogether more than 175 papers are included in the 1958–1959 Antibiotics Annual. The publication of this series on antibiotics constitutes an outstanding contribution and an important and convenient source of information of value to research workers and to others interested in the fascinating subject of antibiotics.

Cahiers de Synthèse Organique. Vol. V. By JEAN MATHIEU and ANDRÉ ALLAIS. Masson et Cie., Paris, 1959. 394 pp. 15 x 22 cm. Price paperbound, 7,800 Francs; cloth, 8,500 Francs.

Volume V of this series (in French) is concerned with molecular degradations. The first section (numbered 12) deals with the loss of a functional carbon. The second section (numbered 13) takes up the cleavage of carbon chains and the rupture of carbocycles. The style of presentation is similar to that in Vol. IV which was described in THIS JOURNAL, **47**, 836(1958).

Staphylococcus Pyogenes and Its Relation to Disease. By STEPHEN D. ELEK. E. S. Livingstone Ltd., Edinburgh and London, 1959. Exclusive U. S. Agents, The Williams & Wilkins Co., Baltimore. vii + 767 pp. 15 x 24 cm. Price $15.

This book is a monograph on *Staphylococcus pyogenes* and its relation to disease. This relation has been emphasized dramatically by the antibiotic-resistant infections due to this microorganism in many hsopitals. The text includes chapters on problems of taxonomy, bacteriological techniques, metabolism, serological studies, virulence and disease, action of combatting agents, and clinical problems. A bibliography of publications covers 188 pages. Subject and author indexes are appended.

Six-Membered Heterocyclic Nitrogen Compounds With Three Condensed Rings. By C. F. H. ALLEN. Interscience Publishers, Inc., New York, 1958. xxii + 624 pp. 15 x 23 cm. Price $26 single; $24 subscription.

This is the twelfth volume of a series of monographs devoted to a modern, detailed, and comprehensive presentation of heterocyclic chemistry, each prepared by a group of authorities. The subdivisions have been designed to cover the field in its entirety by monographs which reflect the importance of the interactions of the various compounds.

This volume covers polynuclear heterocyclic compounds containing nitrogen and three fused six-membered rings. These compounds are not only of unusual theoretical interest, but of considerable practical importance to the drug industry. The arrangement and the discussion of the various classes emphasize their similarity and significant differences. The literature has been covered through 1952. The book is divided into nine general sections, the subject matter of which is arranged as follows: I: Azaanthracenes; II: Azaphenanthrenes; III: 4-Azaphenanthrenes; IV: 5-Azaphenanthrenes; V: Diazaphenanthrenes (except Phenanthrolines); VI: 1,10-,1,7-, and 4,7- Diazaphenanthrenes; VII: Other polyazaphenanthrenes; VIII: Azabenzonaphthenes; IX: The ultraviolet absorption spectra of polycyclic heterocyclic aromatic compounds.

The "a" system of nomenclature has been used as in earlier volumes. All individual chemical substances, however, are also named according to the system employed by *Chemical Abstracts*, and the index includes all names.

The editor and the contributors seem to have accomplished their aim in writing this treatise, and the subject has been treated in a most competent and scholarly manner.

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